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Chemical-releasing substrates that allow the effects of certain pollutants on attached algal communities to be studied under natural stream conditions without impacting the stream ecosystems were developed. Several investigations in both artificial streams and in the field were conducted with these substrates; in them algal communities were first allowed to colonize and develop on the diffusing surface of these substrates for several weeks, after which time toxicant solutions were added to the flasks and allowed to diffuse through the attached algal communities. Laboratory studies revealed that the responses of algal communities to substrate-released copper were not different from responses generated in traditional toxicity tests. The experimental substrates may be useful in validating the results of laboratory toxicity tests under natural environmental conditions, confirming the results of upstream-downstream pollution studies, which are flawed by the lack of rigorous controls, and measuring the variation in algal community sensitivity to stress. Field studies focussing on the last application failed to reject the null hypothesis that communities in polluted streams respond to copper differently from those in unpolluted streams, but they were flawed by unpredicted methodological problems.

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ABSTRACT

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SECTION I

Validation of a New Method for Delivering Toxicants to Periphyton Communities in Streams

Introduction

Water quality criteria that have been established to help protect valuable, aquatic ecosystem services and commodities are largely derived from the results of laboratory toxicity tests performed on different species in isolation from one another (Stephan et al., 1985). This approach is indispensable for initially assessing the environmental hazards of the great number of toxic substances discharged into aquatic ecosystems today. However, because these tests can fall short of generating reliable predictions of toxic impacts to interacting populations and communities of organisms in aquatic ecosystems, investigations at higher levels of ecological organization are needed to validate single species toxicity tests (Cairns, 1983; Odum, 1984; Kimball and Levin, 1985; Schindler, 1987; Cairns and Niederlehner, 1992).

Studies of the effects of copper on natural lentic ecosystems have been relatively common due to the use of copper sulfate as an algicide for controlling nuisance algal blooms (e.g., Whitaker et al., 1978; Effler et al., 1980; McKnight, 1981). These studies have provided some useful observations of the responses of lake ecosystems to copper. However, they are not controlled experiments

-- often the natural fluctuations of measured endpoints are not adequately estimated either before treatment or concurrently, in suitable reference sites, so toxic responses cannot be conclusively determined (e.g., Effler et al., 1980). Nevertheless, observed lake responses to copper additions have provided preliminary evidence that the responses of natural systems to toxicants often cannot be predicted from laboratory bioassays. For example, toxicity tests did not demonstrate that populations of a copper sensitive, nuisance dinoflagellate species in Mill Pond in eastern Massachusetts would contain a significant number of tolerant individuals (McKnight, 1981).

Controlled field mesocosm studies and lake manipulations that have more extensively established the normal operating ranges of measured ecosystem parameters have yielded more informative estimates of lentic ecosystem response to toxic stress. Moore and Winner (1989) demonstrated that seven-day, laboratory toxicity tests with Ceriodaphnia dubia predicted the subsidizing effect that copper had on the abundance of a closely related zooplankter in limnocorrals in Brandenburg Pond, Ohio, but these tests underestimated the adverse responses of other organisms to copper. Using copper treated field mesocosms in East Twin Lake, Ohio, Havens (1994) confirmed Odum's (1985) hypothesis that anthropogenic stress reverses the direction of autogenic succession. Phytoplankton in experimental lakes in northwestern Ontario responded to a variety of chemical stresses by shifts in community composition, but resistant species reproduced rapidly enough to

maintain ecosystem functions such as primary productivity and nutrient cycling (Schindler, 1987).

Manipulative studies in streams and rivers pose an additional complexity: toxicant additions to these systems are not contained and may inevitably affect downstream reaches not being investigated. In an early example of a whole stream manipulation, laboratory tests fairly accurately predicted the mortality of fish in Shayler Run exposed to 120 $\mu g \cdot l^{-1}$ Cu for nearly three years, but they were not complex enough to predict fish avoidance of copper, which adversely affected spawning activity in dosed reaches of the stream (Geckler et al., 1976). Leland and Carter (1984) described the copper-induced species replacements that occurred in a Sierra Nevada, experimental stream system receiving up to 10 $\mu g \cdot l^{-1}$ Cu for one year, resulting in the maintenance of algal biomass at unexposed levels. Recently, submerged in-stream channels allowed the characterization of an increased ability of microbial communities to degrade a quaternary ammonium surfactant after being exposed to it for ten days (Shimp and Schwab, 1991). The in situ channels minimized dilution of the test chemical and allowed the investigators to conduct this experiment using less toxicant than would have been required in a whole-stream manipulation.

A variety of nutrient-releasing substrates (e.g., permeable clay pots filled with nutrient enriched agar) have been developed to allow replicated in situ studies of the nutrient limitations of periphyton communities without enriching entire aquatic ecosystems (Pringle and Bowers, 1984; Fairchild et al., 1985; Lowe et al.,

1986; Gibeau and Miller, 1989). We have modified this technique to allow a range of concentrations of toxicants to be delivered to replicate, lotic periphyton communities using toxicant-releasing substrates, while preventing adverse effects on the streams under investigation. This research was conducted to validate the performance of toxicant-releasing substrates in laboratory artificial streams.

Copper was used as a model toxicant in validating the toxicant-releasing substrates because its effects on algal species and communities are fairly well documented. Copper has been shown to inhibit many physiological processes of algae, including: (1) photosynthesis (Steemann Nielsen and Wium-Andersen, 1971; Cedeno-Maldonado and Swader, 1974; Stauber and Florence, 1987), via impairment of both photosystem I (Droppa and Horváth, 1990) and photosystem II (Renganathan and Bose, 1990); (2) respiration (Cedeno-Maldonado and Swader, 1974), which may be less sensitive than photosynthesis (Cedeno-Maldonado and Swader, 1974; Leland and Kuwabara, 1985; but see Stauber and Florence, 1987); (3) mitosis, via reaction with glutathione (Stauber and Florence, 1987), which often results in an increase in cell size (Foster, 1977; Lumsden and Florence, 1983; Visviki and Rachlin, 1994); (4) nutrient uptake (Peterson and Healey, 1985); and (5) catalase activity (Stauber and Florence, 1987). The effects of copper on some of these and other processes have also been described at the community level (e.g., Goering et al., 1977; Harrison et al., 1977; Hedtke, 1984; Leland and Carter, 1985; Sugiura, 1992). Copper often affects community

structure by shifting (increasing and decreasing, respectively) the relative abundances of tolerant and sensitive algal species and by causing a decrease in species richness (Thomas and Seibert, 1977; Weber and McFarland, 1981; Leland and Carter, 1984; Oliveira, 1985; Meador et al., 1993). Bluegreen algae have been purported to be the most copper sensitive algae (Oliveira, 1985; Lüdernitz and Nicklisch, 1989; Meador et al., 1993), but this group contains some relatively resistant taxa as well (Effler et al., 1980; Weber and McFarland, 1981; Leland and Carter, 1984; Oliveira, 1985). In addition, heterotrophic bacteria often become more abundant in algal communities exposed to moderate levels of copper (but see Effler et al., 1980), presumably due to an increase in organic carbon released by stressed or dead algal cells and or a reduction in control by bacterial consumers (Vaccaro et al., 1977; Hedtke, 1984; Sugiura, 1992; Havens, 1994). Finally, the influence of physicochemical factors (Klotz, 1981; Peterson and Healey, 1985; Sprague, 1985; Brezonik et al., 1991) and the influence of algal mechanisms of extracellular modification (Steemann Nielsen and Wium-Andersen, 1971; Foster, 1977; McKnight and Morel, 1979; Lumsden and Florence, 1983; Gekeler et al., 1988; Meador et al., 1993) and intracellular detoxification (Silverberg et al., 1976; Stokes et al., 1977; Twiss and Nalewajko, 1992) on the bioavailability and toxicity of copper have been well characterized.

In this study, periphyton communities developed on toxicant-releasing substrates in laboratory artificial streams were exposed

to copper in two ways. Either soluble copper was added dropwise at known rates to the water columns of flow-through streams, or it was delivered through the toxicant-releasing substrates and into the periphyton communities growing on the substrates. Periphyton community response and recovery were assessed in a summer and a winter experiment. The objective of this study was to elucidate any effects that the new method of toxicant delivery (i.e., exposure to substrate-released toxicant) might have on periphyton community response to copper.

Materials and Methods

Toxicant-Releasing Substrate and Artificial Stream Designs

Toxicant-releasing substrates were constructed from 750 ml polystyrene tissue culture flasks (Falcon) and 4.2 mm thick, unglazed terra-cotta clay tiles fired at 1062 °C (Fig. 1). 10 cm x 10 cm square holes were cut in the flasks, over which the clay tiles were sealed with silicone sealant (Dow Corning). Completed substrates had an exposed tile area of 106 cm^2 and contained a volume of 790 ml. Preliminary experiments with these substrates demonstrated that the flux of several aqueous, inorganic toxicants (e.g., sodium hypochlorite, sulfuric acid, zinc sulfate, and cupric chloride) from the toxicant-releasing substrates is proportional to the toxicant concentration in the modified flasks (unpublished data).

An artificial stream system consisting of nine flow-through channels (Fig. 2) was constructed for investigating the efficacy of these substrates in lotic ecotoxicological investigations. A flow velocity of 10 $cm \cdot s^{-1}$ was established in each of the individual 95 cm (long axis) x 70 cm (short axis) x 19 cm (depth) elliptical, fiber glass channels by means of a rotating paddle wheel. Carbon-dechlorinated, municipal water, derived from the New River (Montgomery Co., Virginia, U.S.A.), flowed from the headbox into each stream at a rate of 1.0 $l \cdot min^{-1}$. The water left each stream through a 12.5 cm high standpipe that served to retain 58 l of circulating water in each channel (≈1 hr. retention time). The toxicant-releasing substrates were secured in place by means of velcro strips fastened to the back of the substrates and to the bottom of the channels with waterproof epoxy resin. A baffle, positioned upstream of the toxicant releasing substrates, minimized differences in flow velocity across the substrates. Light was provided by two 1.22 m Vita-lite full-spectrum bulbs (color rendering index > 90, Durotest Corp.), yielding a photon flux density at the water surface of 47.5 $\mu E \cdot m^{-2} \cdot s^{-1}$. Light and dark regimes approximated the local, natural photoperiods occurring at the beginning of each experiment.

Periphyton covered rocks collected from two sites in the New River (Pembroke, Virginia and McCoy, Virginia) were placed in the headbox as a source of microbial propagules in the water entering the artificial streams. These rocks were replaced weekly during experiments in this system. Macroinvertebrate grazers on the rocks

were excluded from the influent water by means of 0.23 mm mesh screen covering the headbox outflow pipe.

Experimental Design and Data Collection

Two toxicity tests with $CuCl_2 \cdot 2H_2O$ were conducted in the artificial streams. In each experiment periphyton communities that had developed on toxicant-releasing substrates in the absence of stress were exposed to copper in two ways: (1) solutions of $CuCl_2 \cdot 2H_2O$ were added dropwise to the water columns of four streams with the use of peristaltic pumps in a manner that enabled total water column copper concentrations in the streams to be maintained at predetermined levels; and (2) solutions of $CuCl_2 \cdot 2H_2O$ were added to toxicant-releasing substrates in four additional streams in order to expose the periphyton communities to substrate released copper.

The two experiments were begun on September 4, 1992 (photoperiod 13L:11D) and on December 26, 1992 (10L:14D) by placing six, uncolonized toxicant-releasing substrates filled with filtered (0.2 μm pore size) stream water in each stream. Because water temperature (Table I) and photoperiod were significantly different at these times and because propagule communities were taken from the New River during different seasons, the two laboratory experiments were designated summer and winter tests. Colonization by headbox propagules and subsequent periphyton growth on the substrates was allowed to proceed for 365 light hours (i.e., approx. four weeks in the summer and five weeks in the winter) in

the absence of copper stress. During this time frequent measurements of stream temperature and pH were made with an Acumet 1003 portable pH meter and a KCl/AgCl probe with automatic temperature compensation (Fisher Scientific), and conductivity was measured with a YSI Model 33 S-C-T conductivity meter. The performance of the carbon dechlorinator was assessed by monitoring the free and total chlorine concentrations in the stream water using amperometric titrations with a Wallace and Tiernan Titrator. Hardness and alkalinity were determined in unfiltered samples using EDTA and sulfuric acid titrations (endpoint pH=4.50), respectively (APHA et al., 1989). Additional physicochemical parameters were measured less often. Dissolved reactive orthophosphate, ammonium, nitrite, and silica concentrations in samples filtered through 0.45 μm membrane filters were analyzed colorimetrically using standard protocols (APHA et al, 1989). Chloride, nitrate, and sulfate concentrations were determined simultaneously in filtered (0.45 μm) water samples with a Dionex Series 2000i/SP ion chromatograph. In a preliminary investigation it was found that the physicochemical parameters, with the exception of chloride concentration, did not differ due to copper dose levels or the location of individual streams in the laboratory, so physicochemical determinations were restricted to headbox water in these experiments.

At the end of the colonization phase, one randomly selected substrate was removed from each stream for analysis of baseline community structure. Periphyton growing on the clay tile was scrubbed off with a stiff bristle toothbrush, rinsed into a sample

beaker with filtered stream water, brought up to a known final volume, and homogenized for 20 seconds using a Biomixer (Fisher Scientific). Quantitative subsamples were immediately removed for analysis of adenosine triphosphate (ATP) content, chlorophyll a (chl a) content, and total community biomass. First, an aliquot of periphyton homogenate from each baseline sample was filtered onto a 0.2 μm pore size, membrane filter. ATP was extracted from the filtrate in boiling Tris buffer (0.02 M; pH=7.5) for five minutes. The extract was quickly frozen, stored at -20 °C, and later analyzed using the luciferin-luciferase assay (APHA et al., 1989). Second, a known subsample was filtered onto a 1.5 μm mesh, glass fiber filter. This filtrate was macerated with a Potter-Elvehjem tissue grinder (Wheaton), and the chl a it contained was extracted into cold, 90% buffered acetone overnight in the dark at 4 °C. On the following day chl a content was determined spectrophotometrically, with pheophytin a correction (APHA et al., 1989). Finally, an aliquot of periphyton homogenate was taken from each baseline sample, filtered onto a 1.5 μm mesh, glass fiber filter, and dried at 105 °C for 24 hr. Periphyton biomass (i.e., ash-free dry mass) was determined in these samples as the loss of mass upon combustion at 500 °C for 1 hr. (APHA et al., 1989).

After the completion of baseline structural sampling a seven-day copper dosing phase was initiated. The periphyton communities in each of four randomly selected streams received one of four different concentrations of substrate-released copper: the five substrates remaining in these streams were removed, emptied, filled

with solutions of $CuCl_2 \cdot 2H_2O$ in filtered (0.2 μm) stream water, and replaced to their original positions in the streams. Manipulating the toxicant-releasing substrates in this manner resulted in no visible loss of periphyton. 0.50, 1.0, 5.0, and 10 $gCu^{2+} \cdot l^{-1}$ solutions were used in the summer test; these concentration were changed to 0.25, 0.50, 2.0, and 4.0 $gCu^{2+} \cdot l^{-1}$ during the winter test based on summer results. Because addition of extremely concentrated Cu^{2+} solutions to the modified flasks was required to generate toxic effects on periphyton structure, care was taken not to spill the copper solutions on the periphyton communities during substrate manipulation. The water in all five toxicant-releasing substrates in each of four additional, randomly selected streams was replaced with filtered stream water in the same manner in order to control for the effects of substrate manipulation. The water columns of each of these streams received one of four concentrations of copper using a peristaltic dosing apparatus. Nominal water column copper concentrations of 10, 30, 100, and 300 $\mu gCu^{2+} \cdot l^{-1}$ were chosen for the summer test; on the basis of summer results, concentrations of 5, 25, 100, and 1000 $\mu gCu^{2+} \cdot l^{-1}$ were selected for the winter test. The remaining stream served as a reference stream. The contents of the five substrates in this stream were also replaced with filtered stream water, but this stream received neither substrate-released copper nor copper added to the water column. Unfiltered water samples were removed daily from each stream and acidified to a final concentration of 0.15% v/v with metal free nitric acid (APHA et al., 1989). Copper

concentration in these samples were determined by AAS (graphite furnace atomization) with a Perkin-Elmer 110 Atomic Adsorption Spectrophotometer using manufacturer recommended conditions.

To avoid pseudoreplication (Hurlbert, 1984), only one, randomly selected substrate was removed from each stream after one week of copper exposure in order to assess community structural responses. In addition to the structural measurements made on the baseline samples, a quantitative fraction was removed from each periphyton sample, filtered onto a copper free glass fiber filter (1.5 μm mesh size), washed several times with filtered headbox water to remove soluble copper, and dried at 105 °C for 24 hrs. for the determination of copper bioconcentration (i.e., toxicant exposure level). Dried samples were combusted at 500 °C for 1 hr., and copper was extracted from the residual ash in concentrated, metal free nitric acid for one week at room temperature. After the extraction, the samples were diluted to a final concentration of 1% v/v nitric acid and analyzed using AAS. The remaining periphyton slurries were preserved with M3 fixative lacking iodine (APHA et al., 1989). Preserved subsamples were stained with sterile Acridine Orange (0.01% w/v final concentration), filtered onto black, polycarbonate membrane filters (0.2 μm; Poretics Corporation), and de-stained with sterile, distilled water. Direct counts of the filters using epifluorescence microscopy at X1000 magnification (Hobbie et al., 1977) enabled heterotrophic bacterial abundance to be determined. Biovolume was estimated from average bacterial dimensions, and bacterial biomass was calculated from

biovolume using a standard conversion factor (Bratbak, 1985; Edwards et al., 1990). In addition, permanent algal mounts were prepared from the preserved samples. Quantitatively diluted subsamples for diatom enumeration were dehydrated by the vapor substitution method and mounted in HYRAX (Custom Research and Development, Auburn, CA) mounting medium (Stevenson and Stoermer, 1981). Duplicate subsamples were also mounted in Taft's Syrup Medium (Stevenson, 1984) to minimize distortion of soft algae and to allow non-diatom algae to be identified and enumerated. The slide mounts were scanned at X1000 with a Nikon Microphot-FX microscope, using DIC microscopy when helpful for diatom identification. For both mount types, 500 specimens were identified (Pryfogle and Lowe, 1979) to the lowest possible taxon using standard taxonomic keys (Desikachary, 1959; Patrick and Reimer, 1966, 1975; Prescott, 1970, 1978; Germain, 1981; Dillard, 1989a, 1989b, 1990, 1991a, 1991b). Diatoms displaying some remnant of protoplast in the HYRAX mounts and chlorophyll bearing diatoms in the TSM mounts were considered to be alive at the time of sample collection and were distinguished from dead (i.e., empty) frustules. Quantitative preparation of algal mounts allowed cell abundances to be measured per unit substrate area. Algal species richness (no. of live algal taxa in 500 cell counts) was calculated in all response phase samples, and samples by species abundance matrices for the exposed communities were compared to the reference stream matrix using Pinkham-Pearson coefficients of similarity (Pinkham and Pearson, 1976). At the end of response phase

sampling, the contents in all substrates remaining in each stream were replaced with filtered stream water.

A randomly selected substrate was sampled from each stream after two weeks of recovery from copper stress. The community structural measurements made after one week of exposure to copper were repeated on samples collected at this point. The three leftover substrates per stream were used in the development of a recirculating chamber that was designed to enable measurement of community functional parameters (data not given here).

Data Analysis

Differences in diluent water quality between the summer and winter tests were assessed by comparing the seasonal means of the physicochemical parameters using two sample t-tests, corrected for unequal variances when appropriate (Zar, 1984; SAS Institute Inc., 1990). Community structural attributes were regressed on copper bioconcentration (dose) to characterized the toxicological effects of copper on the periphyton communities. In all cases, copper bioconcentration was $\log_{10}$-transformed to linearize the responses and reduce variance heterogeneity. The only response variable that required $\log_{10}$-transformation was community ATP content. Analysis of covariance was used to determine the influence of method of copper delivery (substrate-released copper vs. water column copper) and season (summer vs. winter) on periphyton structural responses to copper (Kleinbaum et al., 1988; SAS Institute Inc., 1990). Significant recovery of community structural attributes and

differential recoveries across method of copper delivery were determined using repeated measures models (Zar, 1984; SAS Institute Inc., 1990). Although as many as six dependent variables were analyzed for a given data set, Bonferroni corrections (Kleinbaum et al., 1988) were not made in interpreting the significance of test results because the primary purpose of this investigation was to characterize the influence of substrate toxicant delivery on toxicological outcome and Bonferroni corrections result in conservative hypothesis testing (i.e., a greater probability of inferring no dosing method effect).

Results and Discussion

Water Chemistry and Baseline Community Structure

Several physicochemical properties of the headbox water were different in the summer and winter experiments (Table 1). One of the primary differences was water temperature, the means for which were approximately 20°C in the summer and 8°C in the winter ($p<0.0001$). While pH was significantly lower ($p=0.0401$) in the summer ($\bar{X}_S$+/-S.E. = 7.35+/-0.02) than in the winter ($\bar{X}_W$+/-S.E. = 7.48+/-0.05), the magnitude of the difference was small.

The artificial stream water was relatively soft throughout both experiments -- all individual hardness measurements fell between 37 and 54 mg/L as $CaCO_3$. However, the mean water hardness for the summer toxicity test was approximately 10 mg/L higher than

that found for the winter test ($p<0.0001$). Alkalinity differed by a similar amount during the summer ($\bar{X}_S$+/-S.E. = 38.6+/-0.8 mg/L as $CaCO_3$) and winter ($\bar{X}_W$+/-S.E. = 30.7+/-2.0 mg/L as $CaCO_3$) experiments ($p=0.0006$). The pH, water hardness, and alkalinity in the individual streams did not vary significantly with water column copper concentration (Jay Comeaux, pers. comm.).

Conductivity, a measure of total dissolved ions, was significantly greater ($p<0.0001$) in the summer ($\bar{X}_S$+/-S.E. = 114+/-0.9 μS) than in the winter ($\bar{X}_W$+/-S.E. = 78.6+/-1.1 μS). Mean summer and winter levels of soluble phosphate ($\bar{X}$+/-S.E. = 88.5+/-30 μg/L and 166+/-12 μg/L, respectively) and nitrate ($\bar{X}$+/-S.E. = 1.90+/-0.20 and 3.09+/-0.66 mg/L, respectively) were not significantly different. Nitrite and ammonium were not detected (detection limit = 10 μg/L) during the summer toxicity test, so assays for these ions were not conducted during the winter test. Significantly more soluble sulfate ($p=0.0208$) was present in the stream water in the winter ($\bar{X}_W$+/-S.E. = 22.0+/-4.8 mg/L) than in the summer ($\bar{X}_S$+/-S.E. = 6.20+/-1.5 mg/L).

Free hypochlorite was never detected (detection limit = 0.010 mg/L) during either the summer or the winter experiments. Combined residual chlorine, a much less toxic form of chlorine, was undetectable (detection limit = 0.010 mg/L) in the summer ($n = 19$) in all but two water samples, which contained 0.010 and 0.015 mg/L, but was detected in all 12 measurements in the winter ($\bar{X}_W$+/-S.E. = 0.020+/-0.001 mg/L). The higher concentration of combined residual chlorine in the winter may have been caused by increased

chlorination of the municipal water, and thus, a greater chlorine load on the artificial stream system's dechlorinator, which was repacked with activated carbon before the initiation of each experiment.

Baseline characteristics of the periphyton were assessed just prior to the initiation of the week-long dosing phase. Baseline total biomass was significantly higher ($p<0.0001$) in the summer ($\bar{X}_S$+/-S.E. = 0.852+/-0.0548 mg/cm^2) than in the winter ($\bar{X}_W$+/-S.E. = 0.315+/-0.0312 mg/cm^2). The ratio of chlorophyll a to biomass was also significantly higher ($p=0.0144$) in the summer ($\bar{X}_S$+/-S.E. = 14.7+/-2.29 mgChla/gAFDW) than in the winter experiment ($\bar{X}_W$+/-S.E. = 7.54+/-1.25 mgChla/gAFDW). Thus, phototrophs appear to have made up a greater proportion of pre-disturbance community biomass in the summer than in the winter. On the other hand, the amount of ATP measured per unit biomass in the baseline assemblages was relatively variable ($\bar{X}_S$+/-S.E. = 0.206+/-0.0409 mg/gAFDW; $\bar{X}_W$+/-S.E.= 0.0994+/-0.0200 mgATP/gAFDW). No significant difference between these means could be detected at the 5% level.

Periphyton communities which developed in the artificial streams after 365 light hours of colonization were structurally different in the summer and in the winter. Total biomass (living and dead) was greater in the summer than in the winter, though mean concentrations of soluble reactive phosphate and dissolved nitrate were not significantly different from one another during the two toxicity tests. The artificial stream system receives fairly high levels of soluble nitrogen and phosphorus. Thus, it is likely that

winter periphyton growth was limited by either lower water temperatures or higher levels of combined residual chlorine.

Baseline communities in the summer contained a greater amount of chlorophyll a relative to biomass than those in the winter. Periphyton communities are known to undergo seasonal succession on natural substrates (Neel, 1968) and seasonally-influenced primary succession on introduced substrates (Hoagland et al., 1982), so it is not surprising that the amounts of chlorophyll a relative to community biomass in the summer and winter were different. In fact, because photosynthesis is generally less temperature-dependent than respiration at lower temperatures, Cairns et al. (1975) hypothesized that, in general, decomposition rates in colder aquatic microbial communities may be low relative to production rates, leading to the accumulation of organic matter. An increase in the relative amount of nonliving organic matter in the winter compared to the summer could have been responsible for the decrease in the ratio of chlorophyll a to total biomass observed for the winter baseline communities.

Mean adenosine triphosphate content was highest in the summer. However, because of high variability in baseline ATP measurements, the summer and winter mean ratios of ATP to biomass could not be distinguished from one another. Due to the lability of some of the measured structural parameters (e.g., chlorophyll a, ATP), periphyton removed from one substrate had to be processed completely before removing another substrate from the artificial streams. For this reason, baseline sampling lasted several hours.

Therefore, some of the variability in the ATP measurements may have been due to real diurnal fluctuations in the average community ATP content (Cole et al., 1967; Holm-Hansen, 1973). Although extremely variable in the baseline and recovery samples, community ATP did respond significantly to copper in the winter (discussed below). Indeed, because it is a good indicator of living biomass, the measurement of ATP is often worthwhile in toxicological studies, especially when interpreted in conjunction with other estimates of periphyton community standing crop (Stevenson and Lowe, 1986).

Community Structural Responses to Copper

After one week of exposure in both the summer and the winter, several structural attributes of the microbial communities were significantly affected by copper. The explanatory variable (copper bioconcentration) was $\log_{10}$-transformed to linearize these responses and reduce variance heterogeneity. The only structural response that required $\log_{10}$-transformation was community ATP content. Copper-induced changes in community structure in the summer were reflected by significant linear regressions against $\log_{10}$-transformed copper bioconcentration.

In the summer, changes in community structure after one week of exposure to copper were reflected by decreases in (1) total biomass, measured as the ash-free dry weight ($p=0.0261$; Fig. 3) and (2) the ratio of chlorophyll a to biomass ($p=0.0095$; Fig. 4). The standard logarithm of ATP relative to total biomass decreased

with increasing copper bioconcentration (Fig. 6), but the decrease was marginally insignificant ($p=0.0568$). The standard logarithm of bacterial biomass relative to total biomass (Fig. 5), showed significant increases with copper bioconcentration during the summer experiment ($p<0.05$).

During the winter toxicity test, total biomass ($p=0.0413$; Fig. 3), the relative amount of ATP ($p=0.0067$; Fig. 6), and the relative amount of chlorophyll a ($p=0.0125$; Fig. 4) decreased significantly. The $\log_{10}$-transformed ratio of bacterial biomass to total community biomass increased in response to bioconcentrated copper, although the increase was marginally insignificant ($p=0.0503$; Fig. 5).

Many of the periphyton responses to copper stress in the summer and winter experiments were qualitatively similar to those reported by other investigators. Total community biomass decreased with increasing copper bioconcentration (Fig. 3). However, community biomass after seven days of exposure to the highest concentrations of copper employed in these experiments was approximately the same as the pre-exposure level. Thus, it appears that community production (i.e., colonization and growth) during the seven-day exposure phase was inversely related to copper bioconcentration and that high concentrations of copper halted net community growth.

Copper induced significant decreases in the ratio of chlorophyll a to biomass during the summer and winter toxicity tests. The reduction in the relative amount of this phytopigment may have been caused by adverse responses of attached algae to

copper at the organismal level, such as copper degradation of chlorophyll a (Robinson and Choi, 1989) or a community-level response, such as algal senescence with a concomitant increase in the abundance of heterotrophs (Hedtke, 1984), or a combination of these two processes.

During both experiments relative bacterial abundance increased in response to copper stress, and the adenosine triphosphate content of periphyton communities decreased. However, when the increase in relative bacterial biomass was significant (summer only), the decrease in community ATP was insignificant, and vice versa. Viable bacteria contribute to the total ATP pool of aquatic microbial communities. A significant increase in relative bacterial abundance in response to copper, for example, may have slightly offset the decrease in community ATP content, resulting in the lack of a significant relationship between community ATP content and copper bioconcentration in the summer. This relationship is highly speculative due to the fact that the two nonsignificant linear models (e.g., those involving ATP in the summer and bacterial abundance in the winter) approached statistical significance at the 5% level.

The positive relationship between the relative abundance of bacteria and bioconcentration in the summer was probably the result of the increased availability of reduced carbon in the form of dead or senescing algal cells and not a direct effect of copper. Although a few bacteria may be sensitive to low levels of copper (Bringmann and Kühn, 1980) some bacteria typically found in aquatic

ecosystems can tolerate far higher concentrations of copper (Flemming and Trevors, 1989) than those tolerated by most aquatic eukaryotes (USEPA, 1985).

Summer and Winter Differences in Periphyton Response to Copper

Analysis of covariance revealed significant intercept differences in response curves during the summer and winter experiments (i.e., significant season effects) for community ash-free dry weight ($p<0.0001$; Fig. 3), chlorophyll a ($p<0.0001$; Fig. 4), and bacterial biomass ($p=0.0025$; Fig. 5). Ash-free dry weight and the relative community chlorophyll a content decreased with increasing toxicant concentration during both experiments but were consistently higher in the summer than in the winter across the entire range of copper concentrations. Conversely, relative bacterial biomass increased with copper bioconcentration in both seasons and was consistently greater in the winter across all copper concentrations in the communities. Of these seasonally dependent variables, only ash-free dry weight responded differently (i.e., with different slopes) during the two experiments ($p=0.0489$; Fig. 3).

The season in which the toxicity tests were conducted was an important factor governing the relationships between several of the structural attributes of the periphyton communities and copper bioconcentration. The summer ratio of chlorophyll a to biomass (Fig. 4) at the end of the seven-day exposure phase was greater than the winter ratio across all concentrations of copper.

However, the slopes of the summer and winter relationships were statistically indistinguishable. Although communities contained relatively less chlorophyll a in the winter, perhaps as a result of differential primary microsuccession (Hoagland et al., 1982), the sensitivity of chlorophyll a to copper did not differ in the two experiments. On the other hand, heterotrophic bacteria contributed more to total community biomass in the winter across all copper concentrations (Fig. 5). Again, the slopes of the dose-dependent responses of bacterial biomass are not statistically different during the two experiments, and it is concluded that the increase in bacteria was similarly induced by copper in both seasons.

Total community biomass was greater on an areal basis in the summer than in the winter across all concentrations of copper (Fig. 3). However, the slope of the relationship between total biomass and copper bioconcentration was significantly greater in magnitude in the winter. Although community colonization and growth was allowed to proceed for 365 light hours during both the summer and winter toxicity tests, higher water temperatures in the summer were probably responsible for greater biomass accumulation. When stressed with copper, the summer periphyton communities may have lost relatively more biomass, due to sloughing of cells and dead organic matter, when compared to the more adherent winter communities. Thus, unlike chlorophyll a or bacterial biomass, total community biomass was impaired more in the summer than the winter.

Effects of Method of Copper Delivery on Toxicological Responses

Some of the responses of periphyton communities dosed in a bottom-up fashion via the chemical-releasing substrates appeared to be shifted along the abscissa, as if these assemblages were slightly more tolerant of copper. Specifically, this was observed during the summer experiment for ash-free dry weight (Fig. 3), chlorophyll a (Fig. 4), and perhaps bacterial biomass (Fig. 5). However, none of these apparent shifts were significant ($p>0.29$ for differences in the intercept (i.e., method) term of all ANCOVA models). After accounting for the effects of method, alone, there were no significant differences in slopes (i.e., method-by-copper bioconcentration interactions).

An explanation for this apparent shift, whereby communities on chemical-diffusing substrates demonstrated responses similar to communities in water column dosed streams but at slightly higher copper exposure levels, may be explained by a methodological artifact. Removing periphyton from the substrates inevitably resulted in the removal of a small amount of clay, as dust, from the terra-cotta tiles. In the summer, clay that was removed from the outer porous surface of the chemical-releasing substrates after all the periphyton had been removed contained two orders of magnitude more abiotically bound copper. This additional clay bound copper was incorporated into the measurements of copper bioconcentration and resulted in slight overestimates in periphyton metal accumulation.

In general, the summer and winter structural responses of periphyton communities to substrate-released copper agree with previously published results and cannot be statistically distinguished from those of communities dosed through the water column. This important result indicates that no major methodological artifacts hinder the application of chemical-releasing substrates in studies of the effects of copper (and perhaps other inorganic toxicants) on periphyton communities in natural streams.

Periphyton Recovery from Copper Stress

Structural measurements were made on periphyton communities immediately following one week of exposure to stress and on communities from the same artificial streams after two weeks of recovery. Analysis of these data was performed using a repeated measures model. The single measurement of copper bioconcentration made at the end of the seven-day exposure period was used for each pair of exposure phase and recovery phase responses. The analysis revealed significant effects of the time of sampling (i.e., at the end of a seven day exposure period vs. after a two week recovery period with no additional copper inputs) on several structural responses of the microbial communities.

Total community biomass generally increased two weeks after the termination of stress in the summer ($p=0.0099$) and winter ($p=0.0021$). However, the greatest increase in biomass following the removal of stress occurred at low copper bioconcentration (Fig.

3). This was especially true during the winter experiment, for which this analysis revealed a significant interaction between sampling period and copper bioconcentration ($p=0.0151$) -- in other words, a significant change in slope after two weeks of recovery (Fig. 3). Thus, after two weeks community biomass had not measurably recovered from stress across all concentrations of copper. Measured quantities of ATP in the microbial communities were extremely variable two weeks after the copper stress (data not plotted). Thus, no significant effect of sampling time or higher order interactions with sampling time were found for this response.

The changes in the relative amount of chlorophyll a in the periphyton communities after two weeks of recovery were different during the two experiments (Fig. 4). The amount of chlorophyll a in the summer communities decreased after termination of the copper stress ($p=0.0047$) but increased in the winter ($p=0.0034$) relative to communities stressed with copper for one week. Again, apparent recovery occurred primarily at low and intermediate concentrations of copper and not at the highest concentrations. The slope of the summer relationship between chlorophyll a and $\log_{10}$-transformed bioconcentration at the end of the seven-day copper exposure phase was statistically indistinguishable from the slope of the same relationship after two weeks of recovery (i.e., the interaction between phase and bioconcentration was not significant). In the winter experiment, however, the slope of the linear relationship between community chlorophyll a content and $\log_{10}$-transformed copper bioconcentration after two weeks of recovery was significantly

greater ($p=0.0236$) than that at the end of the seven-day exposure phase. The reason for this change in slope appears to be a lack of increase in the ratio of chlorophyll a to biomass in communities dosed with high (as opposed to low and intermediate) levels of copper stress during the winter exposure phase.

Table 2 lists the concentration of copper in periphyton communities at the end of the seven-day exposure period and in similarly exposed communities after two weeks of recovery. Although communities lost copper during the recovery phase, the communities treated with high concentrations of this toxicant retained significant copper (>1 milligram per gram of total biomass) after two weeks. This retention of copper may have been responsible for the delay in the recovery of total biomass and the chlorophyll a content of communities previously exposed to high concentrations of toxicant.

Although both the substrate-dosed periphyton assemblages and the clay tiles of the toxicant-releasing substrates may have retained more copper than communities and tiles in the water column dosed streams (Table 2; Fig. 7), no significant effects of copper delivery method on community recovery were observed. The novel substrates appear to allow the recovery of aquatic microbial assemblages from copper stress to proceed in a manner indistinguishable from the recovery of communities exposed to copper in the water column.

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TABLE 1. Physical and chemical characterization of the diluent water. Table entries are means, standard errors, and number of observations. Significant differences between summer and winter means are indicated with *, **, and *** for $\alpha<0.05$, $\alpha<0.001$, and $\alpha<0.0001$, respectively. Analyte concentrations below the limit of detection are indicated by b.d.

Characteristic	Summer Toxicity Test	Winter Toxicity Test
Temperature (°C)	20.8 (0.1) *** n=31	8.3 (0.2) *** n=16
pH (units)	7.35 (0.02) * n=16	7.48 (0.05) * n=10
Hardness ($mg \cdot l^{-1}$) as $CaCO_3$	49.1 (0.8) *** n=11	38.0 (0.8) *** n=4
Alkalinity ($mg \cdot l^{-1}$) as $CaCO_3$	38.6 (0.8) ** n=11	30.7 (2.0) ** n=4
Conductivity ($\mu S \cdot cm^{-1}$)	113.7 (0.9) *** n=19	78.6 (1.1) *** n=8
ortho-PO_4^{3-} ($\mu g \cdot l^{-1}$) (dissolved, reactive)	88.5 (29.8) n=4	166 (12) n=4
NO_3^- ($mg \cdot l^{-1}$)	1.90 (0.20) n=4	3.09 (0.66) n=4
NO_2^- ($\mu g \cdot l^{-1}$)	b.d. n=4	not assayed
NH_4^+ ($\mu g \cdot l^{-1}$)	b.d. n=4	not assayed
SO_4^{2-} ($mg \cdot l^{-1}$)	6.20 (1.54) * n=4	22.0 (4.85) * n=4
SiO_2 ($mg \cdot l^{-1}$)	6.02 (0.32) n=2	7.05 (0.55) n=2
Cl^- ($mg \cdot l^{-1}$)	1.61 (1.29) n=2	4.13 (1.19) n=4
Fl^- ($mg \cdot l^{-1}$)	1.37 (0.42) n=2	0.65 (0.20) n=4
Free Residual Chlorine ($mg \cdot l^{-1}$)	b.d. n=19	b.d. n=12
Total Residual Chlorine ($mg \cdot l^{-1}$)	0.001 (0.001) *** n=19	0.020 (0.001) *** n=12

TABLE 2 - Copper bioconcentration by laboratory periphyton communities during summer and winter toxicity tests. Data are reported for the bioconcentration after seven days of exposure to copper and the bioconcentration in similarly exposed communities after two weeks of recovery. "WC" indicates the average copper concentration delivered to the communities through the water column, and "CD" indicates the initial copper concentration placed in chemical-diffusing substrates.

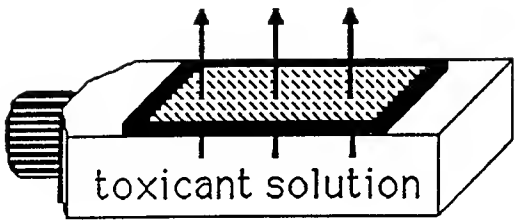
SUMMER

	Cu Bioconcentration (mg Cu/g AFDW)	
Treatment	Just After Exposure	After Two Weeks of Recovery
Reference stream	0.150	0.0802
WC 7.2 µgCu/L	0.694	0.170
WC 20.3 µgCu/L	0.994	0.152
WC 69.1 µgCu/L	4.89	0.480
WC 211.2 µgCu/L	14.2	1.05
CD 0.50 gCu/L	0.907	0.825
CD 1.0 gCu/L	5.51	1.35
CD 5.0 gCu/L	20.0	15.8
CD 10 gCu/L	251	160

WINTER

	Cu Bioconcentration (mg Cu/g AFDW)	
Treatment	Just After Exposure	After Two Weeks of Recovery
Reference stream	0.241	0.267
WC 3.6 µgCu/L	0.481	0.296
WC 14.6 µgCu/L	1.64	0.451
WC 66.1 µgCu/L	9.44	0.833
WC 641.8 µgCu/L	227	7.56
CD 0.25 gCu/L	0.309	0.464
CD 0.5 gCu/L	2.36	1.23
CD 2.0 gCu/L	9.71	5.80
CD 4.0 gCu/L	36.5	10.8

FIGURE 1



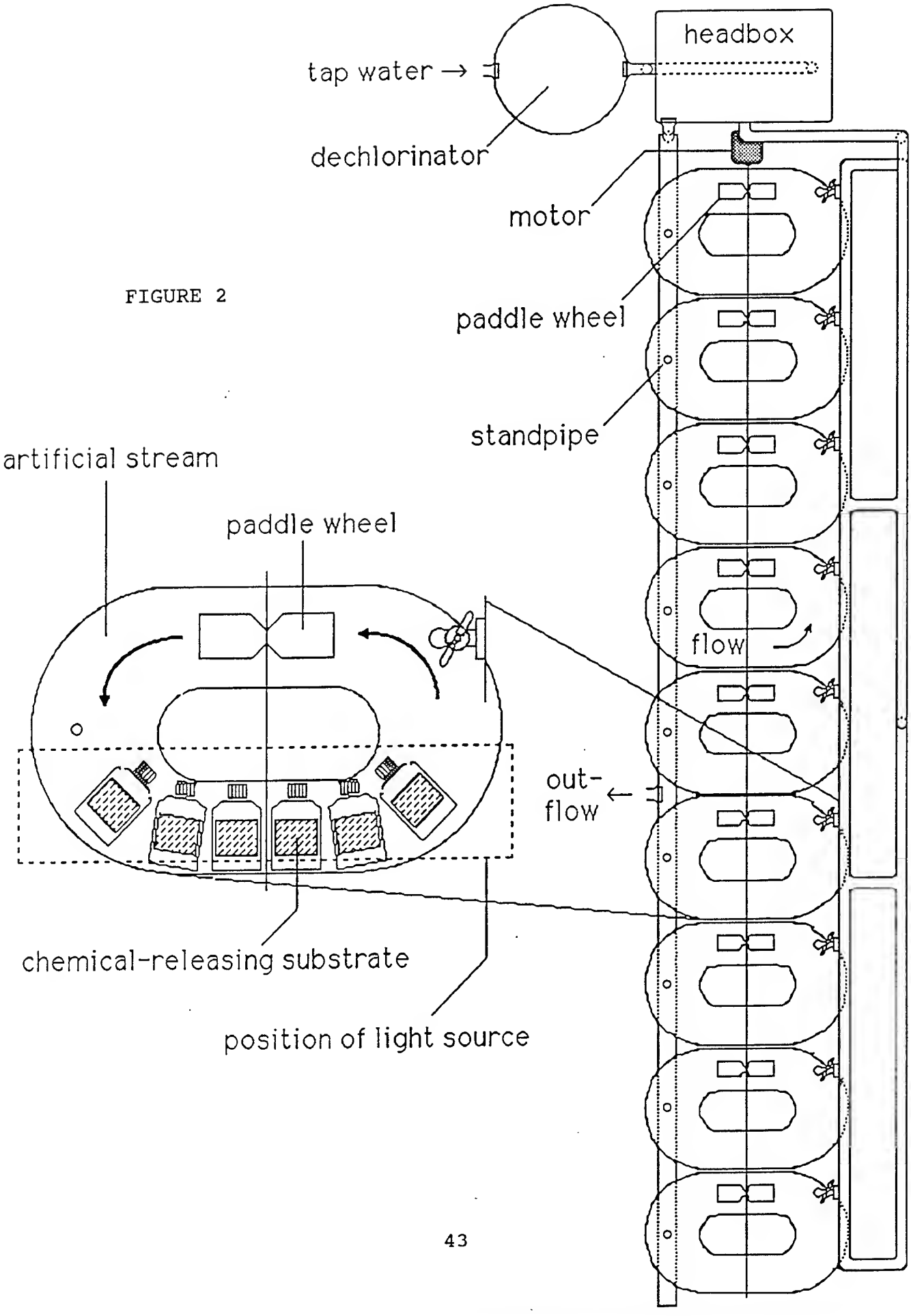

tap water →
headbox
dechlorinator
motor
paddle wheel
standpipe
artificial stream
paddle wheel
flow
out-
flow
chemical-releasing substrate
position of light source

FIGURE 2

FIGURE 3

ASH-FREE DRY MASS vs. Cu BIOCONCENTRATION

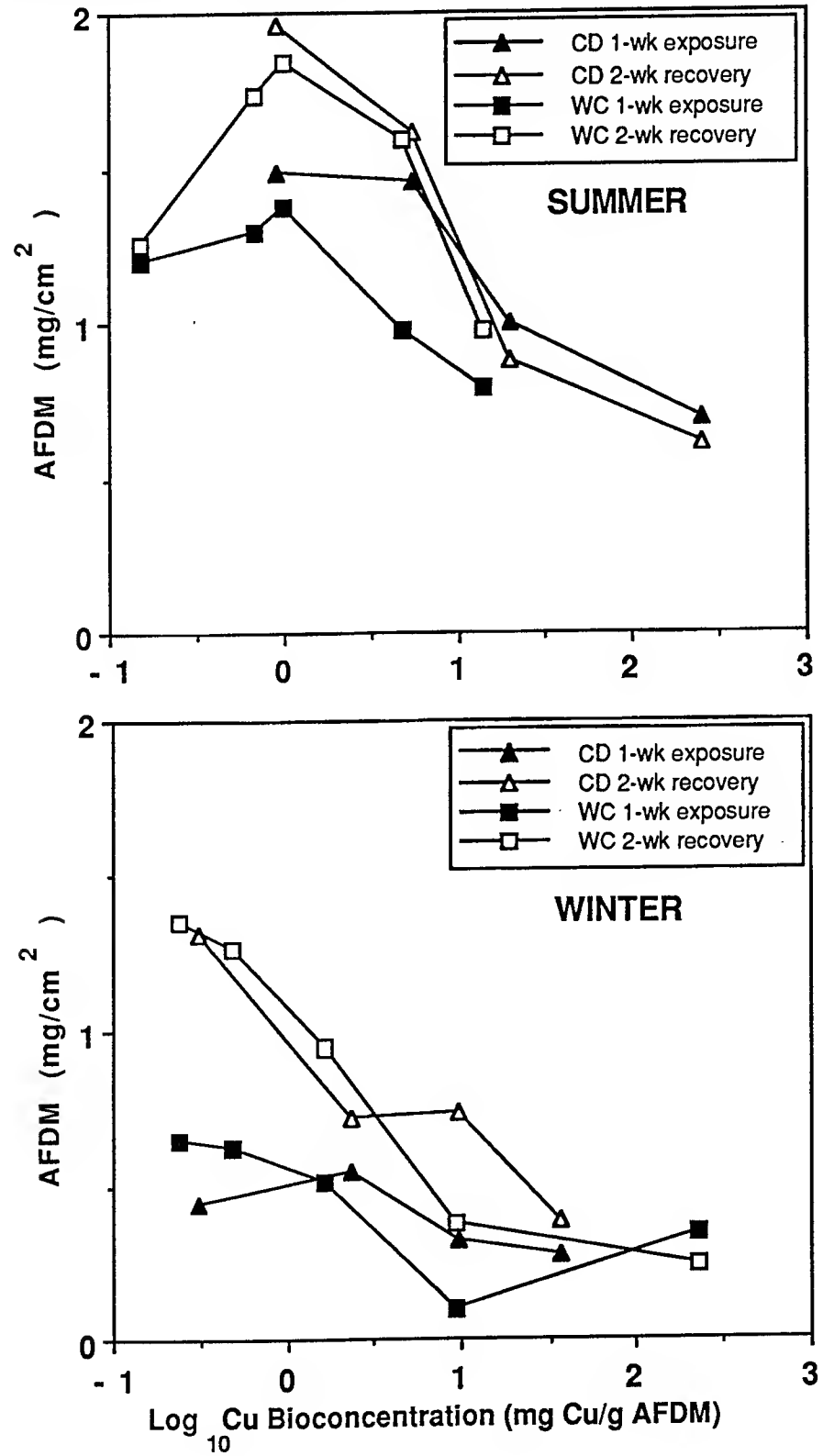


FIGURE 4

CHLOROPHYLL a vs. Cu BIOCONCENTRATION

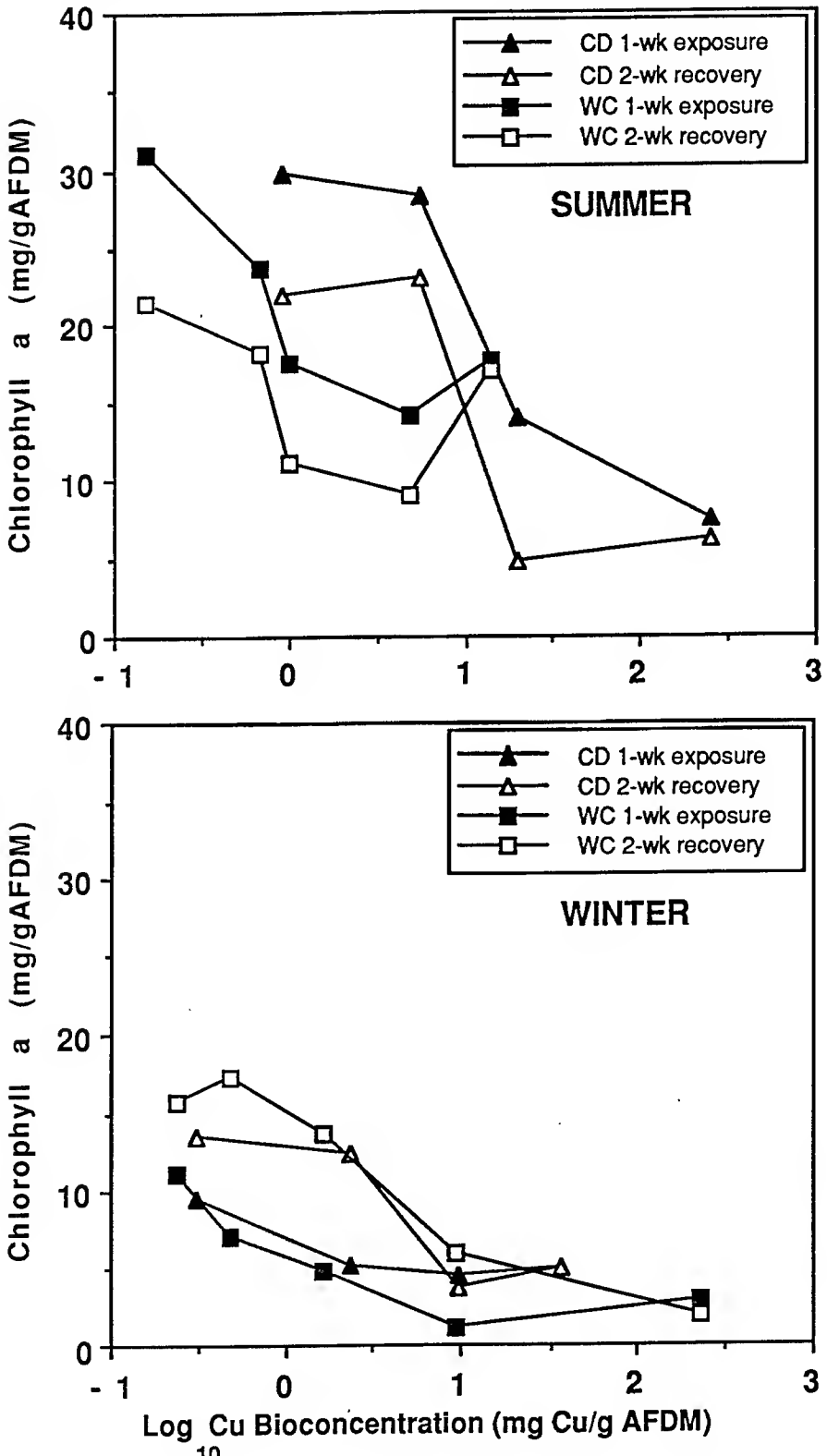


FIGURE 5

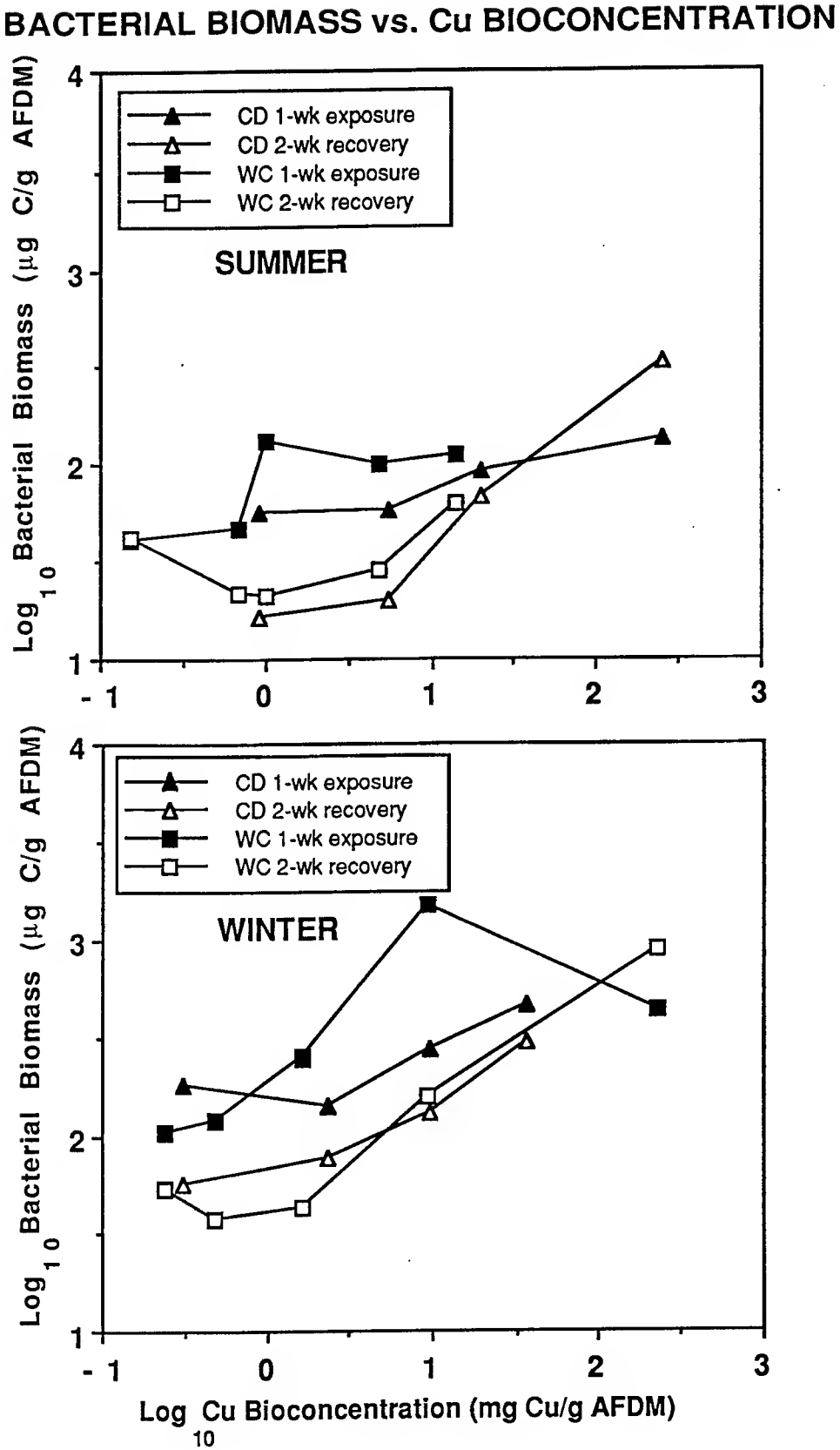

BACTERIAL BIOMASS vs. Cu BIOCONCENTRATION
CD 1-wk exposure
CD 2-wk recovery
WC 1-wk exposure
WC 2-wk recovery
SUMMER
WINTER
Log10 Bacterial Biomass (µg C/g AFDM)
Log10 Cu Bioconcentration (mg Cu/g AFDM)
4
3
2
1
-1
0
1
2
3

FIGURE 6

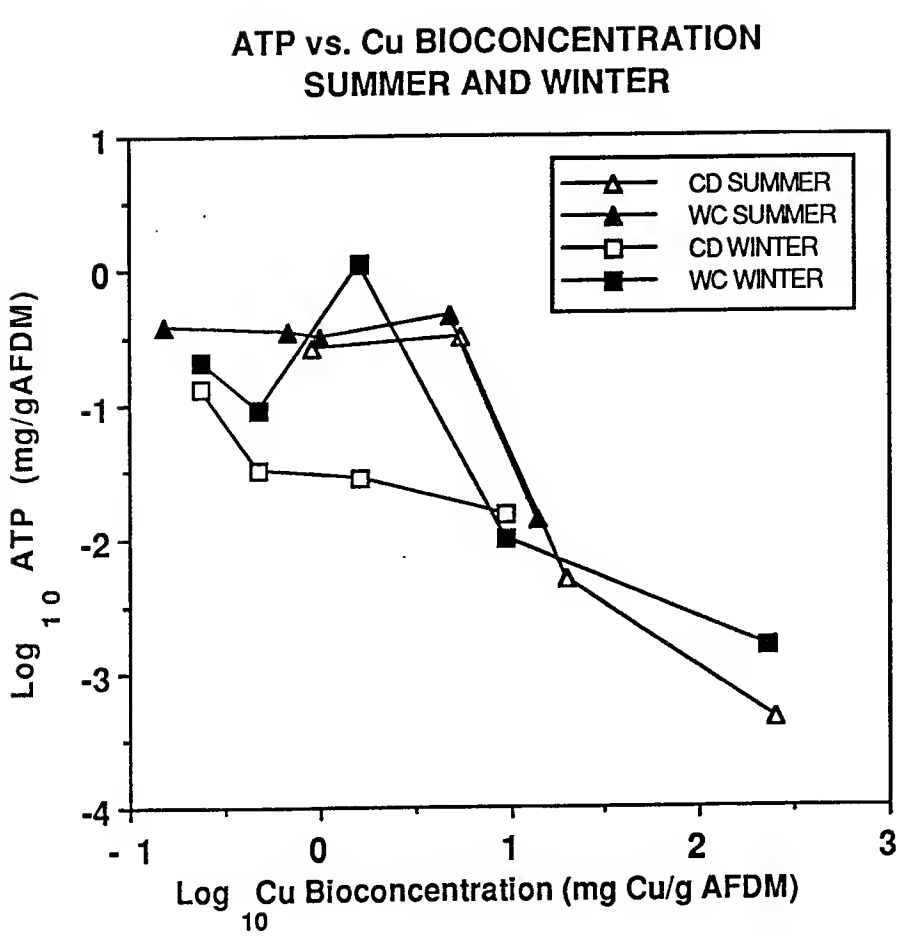
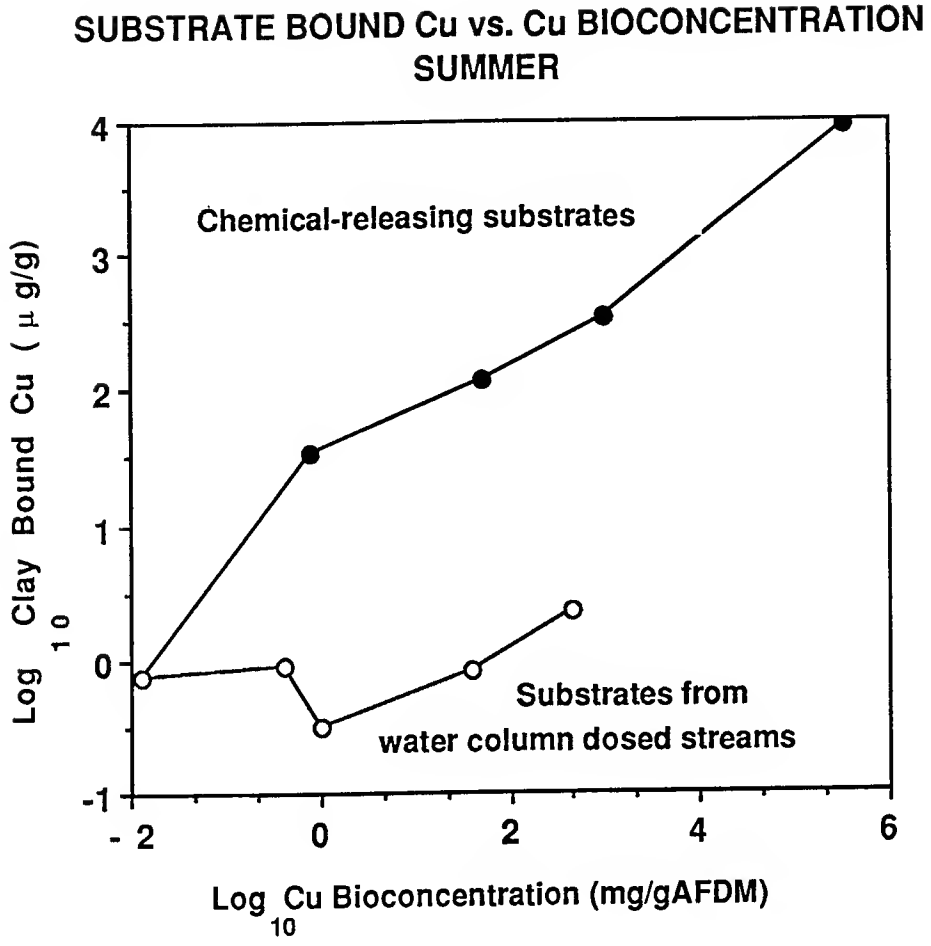

ATP vs. Cu BIOCONCENTRATION
SUMMER AND WINTER
CD SUMMER
WC SUMMER
CD WINTER
WC WINTER
Log 10 ATP (mg/gAFDM)
1
0
-1
-2
-3
-4
- 1
0
1
2
3
Log 10 Cu Bioconcentration (mg Cu/g AFDM)

FIGURE 7


SUBSTRATE BOUND Cu vs. Cu BIOCONCENTRATION
SUMMER
Chemical-releasing substrates
Substrates from
water column dosed streams
Log 10 Clay Bound Cu (µ g/g)
Log 10 Cu Bioconcentration (mg/gAFDM)
4
3
2
1
0
-1
- 2
0
2
4
6

SECTION II

Tolerance of Periphyton Communities to Copper in Unimpacted and Metal Contaminated Streams: the Role of Stress History

Introduction

For several decades, it has been known that history of stress plays an important role in the response of algal populations to toxicants (Kellner, 1955; Stokes, Hutchinson & Krauter 1973; Fisher, 1977; Luoma, 1977). Algae and other organisms may develop tolerances to toxicants and other environmental stressors through physiological acclimation, genetic adaptation, or a combination of these processes. Tolerance via physiological acclimation (e.g., Chapman, 1985) is induced within a single generation and is not passed on to offspring. Genetic adaptation to stress confers tolerance that is inherited by subsequent generations and is not immediately lost in the absence of stress. Genetically-induced tolerance may, however, be rapidly selected against in the absence of stress due to the metabolic cost of its maintenance (Mulvey & Diamond, 1991). In addition, because mechanisms of physiological acclimation also have a genetic basis, acclimation and adaptation are not always distinct processes (Mulvey & Diamond, 1991).

Several studies have illustrated that algal populations often become tolerant to metals and other toxicants to which they are exposed and that the tolerance is often heritable (Stockner &

Antia, 1976; Stokes & Dreier, 1981; Foster, 1986; Kuwabara & Leland, 1986; Wang, 1986; Klerks & Weis, 1987; Blanck, Wängberg & Molander, 1988). The relative contribution of inducible physiological mechanisms (i.e., acclimation) to tolerance is difficult to assess in these studies (e.g., Wang, 1986) due to the fast reproductive rates of algae (but see Stokes & Dreier, 1981). Some authors point to rapid generation time as one reason that algae develop tolerances to toxicants more readily than aquatic macrophytes (deNoyelles et al., 1989) and animals (Klerks & Weis, 1987; Weis & Weis, 1989; Mulvey & Diamond, 1991). Cotolerance -- tolerance to more than one toxicant within a class of pollutants that is induced by exposure to a single toxicant -- may also occur in some algae (Stokes & Drier, 1981; Foster, 1986).

There are several mechanisms by which historically perturbed communities of organisms may develop tolerance to stress. (1) Populations within communities exposed to toxicants may develop tolerance via acclimation and/or adaptation, resulting in an increase in the resistance of community level processes to stress (Blanck, Wängberg & Molander, 1988). (2) The replacement of sensitive taxa with resistant ones within a stressed community, i.e., congeneric homotaxis (Hill & Wiegert, 1980), may also allow community function to be maintained and may slow further stress-induced alterations in community structure (Rapport, Regier & Hutchinson, 1985; Blanck, Wängberg & Molander, 1988). (3) Similarly, functional and structural simplification of a community (Odum, 1985) may allow ecological homeostasis to be more easily

maintained under stress (May, 1977; Schindler, 1987). However, it is also conceivable that a loss of functional redundancy could decrease a community's resistance to subsequent stress (Stokes, 1986). (4) Finally, toxic stress can result in an increase in the relative size of community compartments that can bind or degrade toxicants (Rapport, Regier & Hutchinson, 1985; Meador, Taub & Sibley, 1993). However, toxicants sequestered within community or ecosystem compartments can have deleterious effects as well (e.g., through biomagnification). Although cotolerance at the population level implies that a physiological protective mechanism (e.g., a metal binding protein) is efficacious for multiple, chemically-related stressors (e.g., several heavy metals), generalizable tolerance (i.e., cotolerance) at the community level need not function through specific physiological means, as indicated above.

In recent laboratory experiments, periphyton communities developed under zinc stress for 21 days have been shown to be initially impaired but to change less than previously unexposed controls upon subsequent acute exposure to zinc (Niederlehner & Cairns, 1992) or lowered pH (Niederlehner & Cairns, 1993). Biological modifications of solution chemistry (e.g., DOC released from a resistant alga) allowed gnotobiotic microcosms to recover from copper stress (Meador, Taub & Sibley, 1993). On the other hand, lentic microcosm communities exposed to copper concentrations ranging from 4.0 to 420 $\mu g \cdot l^{-1}$ for 32 weeks showed essentially no signs of acquiring tolerance (Hedtke, 1984).

Field experiments have also demonstrated the development of tolerance of phytoplankton productivity to copper after 23 days in marine limnocorrals (Harrison, Eppley & Renger, 1977) and to atrazine after 21 days in lentic mesocosms (deNoyelles et al., 1989). Field investigations of the development of tolerance in natural lotic ecosystems have been more difficult due to the difficulty of controlling toxicant delivery within flowing systems. However, Leland and Carter (1985) demonstrated unchanged algal biomass and recoveries of nitrogenase activity on natural cobbles and unchanged chlorophyll-specific rates of photosynthesis in periphyton communities colonizing glass slides in copper dosed reaches of a stream, and they implied that species replacement was the mechanism by which copper tolerance was acquired in these reaches. Shimp & Schwab (1991) were also successful in demonstrating an increased capacity of microbial communities to degrade a quaternary ammonium surfactant exposed to this compound for ten days in submerged in situ stream channels.

This research surveyed the copper tolerance of periphyton communities in several previously unimpacted and metals-impacted streams in southwestern Virginia in the eastern United States. A recently developed method was employed that allows toxicants to be delivered to periphyton communities in situ without contaminating the stream ecosystems (Arnegard, McCormick & Cairns, in press). The role that stress history plays in modifying the sensitivities of periphyton was investigated by measuring changes in community structure following experimental additions of copper in the field.

Two outcomes were deemed possible in historically impacted streams: (1) the cumulative or synergistic effects the existing stream contaminants (largely metals) and the added copper could have resulted in heightened negative responses relative to reference communities (Hutchinson, 1973; Cairns, Messenger & Calhoun, 1976; Breaek, Jensen & Mohus, 1976; Visviki & Rachlin, 1994); or (2) the acquisition of community-level tolerance by one or more of the mechanisms outlined above could have resulted in decreased sensitivity to copper.

Materials and methods

Study Sites

Five relatively pristine and five metals impacted sites in the Appalachian Ridges and Valleys and the Central Appalachian ecoregions (Omernik, 1986) of southwestern Virginia and West Virginia (U.S.A.) were chosen for this study (Fig. 8). The reference sites were chosen on the basis of catchment land use patterns.

Bottom Creek [B], Virginia, at 37°06'52" latitude and 80°12'20" longitude is a second order stream, calculated using the method of Shreve (Gordon, McMahon & Finlayson, 1992), excluding streams indicated as ephemeral on 1:24,000 U.S.G.S. topographic maps. Bottom creek lies in the Roanoke River drainage basin. The site is located just downstream from the Nature Conservancy's Bottom Creek

Gorge preserve. Upstream from Bottom Creek Gorge, the riparian zone is mostly complete and agricultural land use is light.

The Clinch River [C] site at Pounding Mill, Virginia, (37°05'00" latitude and 81°41'59" longitude) is a fifth order stream (Angermeier and Smogor, 1993) in the Tennessee River drainage basin. The water quality of much of the river is affected by drainage from coalfields and agricultural areas (Zipper et al., 1992) and impacts of urban origin, but the river contains some reaches within or below zones of recovery that are free of major impacts. Although the Tazewell wastewater treatment plant is located approximately 25.8 km upstream from the Pounding Mill site, the assemblage of snails, clams, and mussels at Pounding Mill has recently been found to be quite robust relative to reaches 25.8 - 21.5 km upstream and 20.9 - 24.2 km downstream (Goudreau, Neves & Sheeham, 1993). Pounding Mill Index of Biotic Integrity (IBI) scores were good in 1991 and 1992 (Angermeier and Smogor, 1993), and recently, liver metallothionein content in Rock Bass (Ambloplites ruprestris Rafinesque) was low relative to other sites in the Clinch River drainage (Khosla et al., 1993).

Goose Creek [Go], Virginia, is a third order stream (calculated) at 37°05'52" latitude and 80°13'02" longitude in the Roanoke River drainage basin. The riparian zone at and above the study site on this stream is a robust mixed hardwood community with few breaks. There is light agriculture upstream from the study site.

Kimberling Creek [K], Virginia, is a first order stream

(calculated) located in the Jefferson National Forest at 37°08'48" latitude and 81°04'36" longitude. It is part of the New River drainage basin. The hardwood riparian zone is complete at this site and above it, all the way to the ephemeral headwater tributaries. Analysis of sediment metals after initiation of this study indicated moderately elevated levels of several metals at this site (see below), and it was discovered, upon further investigation of historical land use, that a large, unpermitted dump about 5.0 km upstream was used by local residents until the early 1960's (Bland County Board of Supervisors, Pers. comm.). Therefore this site was not considered a reference site in any of the analyses presented.

The Little River [L], Virginia, is a fourth order stream (Angermeier & Smogor, 1993) at 37°01'25" latitude and 81°44'04" longitude, located in the Tennessee River drainage basin. This stream has a robust riparian zone of mixed hardwoods, and its catchment contains low levels of agricultural production (Zipper et al., 1992; personal observation). Fish IBI assessments yielded a rating of good along most of the Little River (Angermeier & Smogor, 1993), and recently measured Rock Bass liver metallothionein levels were very low (Khosla, 1993).

Impacted sites were selected on the basis of well documented histories of metal stress caused by industrial effluents and/or coalfield runoff.

The East Fork Greenbrier River [Gr] site in West Virginia is located just downstream from a leather tannery. The experimental

substrates at this site were lost in a heavy spate before copper was added to the communities in situ.

The Guest River [Gu] at Coeburn, Virginia, is a fifth order stream (Angermeier & Smogor, 1993) at 36°55'46" latitude and 82°27'40" longitude, located in the Tennessee River drainage basin. The river drains very active coalfields (Zipper et al., 1992; pers. observation) and receives urban runoff from the city of Coeburn and effluent from the Coeburn Norton Wise sewage treatment plant approximately 2.8 km upstream from the study site (Frazier, T., Virginia State Water Control Board, pers. comm.). Fish IBI ratings were poor at this site in 1991 and 1992, and recent Rock Bass liver metallothionein levels were very high, indicating metal stress at this site (Khosla et al., 1993). In addition, the benthic macroinvertebrate community was rated as being moderately impaired in the late spring of 1993 (Virginia Department of Environmental Quality, pers. communication).

The North Fork Holston River [H] site at Hayters Gap, Virginia, (36°49'40" latitude and 81°54'45" longitude) is a fifth order stream (Snyder, 1992) in the Tennessee River drainage basin. The former Olin Corporation plant in Saltville, Virginia, which is located 16.9 km upstream from the Hayters Gap site, lost as much as 34 kg of mercury daily to settling ponds and into the North Fork Holston River from the 1950's until 1972 as a result of the chlor-alkali process (Seivard et al., 1993). The settling ponds continue to discharge mercury into the river, and the site has been designated the Saltville Superfund Site by the United States

Environmental Protection Agency. In 1990, total mercury and methylmercury tissue concentrations in Corbicula fluminea Müller collected as far as 121 km downstream were significantly higher than those in clams collected from upstream, reference sites, and they were highest near the Hayters Gap study site (Seivard et al., 1993). In addition, fish tissue (sampled at the origin of contamination only) and sediment mercury concentrations (sampled as far as 33.4 km downstream) are still elevated as a result of the plant's past activities (Virginia Department of Environmental Quality, pers. communication).

Peak Creek [Pk] is a third order stream (calculated) at 37°02'48" latitude and 80°47'18" longitude and receives effluent from Magnox, Incorporated, a magnetic tape manufacturer listed by the USEPA as a priority toxic discharger (Willis, 1990), as well as urban runoff from the town of Pulaski, Virginia. Peak Creek lies in the New River drainage basin. The benthic macroinvertebrate community was sampled at this site in 1989 and was degraded relative to upstream communities (Willis, 1989). In addition, lead and zinc concentrations in the sediments were recently found to be above the 95th percentiles for sediment metal concentrations in Virginia streams and copper, cadmium, nickel, and selenium sediment concentrations were above the 85th percentiles for these metals (Willis, L.D., Virginia State Water Control Board, pers. comm.).

Peters Creek [Pt], Virginia, is a second order stream (calculated) at 37°02'48" latitude and 80°47'18" longitude, located in the Roanoke River drainage basin. It receives urban runoff from

Roanoke, effluent from Roanoke Electric Steel, and runoff from Norfolk and Western Railway Company, which is located adjacent to the study site. Although Roanoke Electric Steel is listed by the USEPA as a priority discharger (Wills, 1990), no historical information on ecological impairment or water and sediment metals at this site is available (Willis, L.D., Virginia State Water Control Board, pers. comm.).

Surveys of benthic macroinvertebrate communities, habitat quality, and sediment metal burdens were conducted just prior to initiation of the experiment to corroborate the stream categorizations above. Single macroinvertebrates samples were collected at each site using the Rapid Bioassessment Protocol (RBP) II (Plafkin et al., 1989), and the number of Ephemeroptera, Plecoptera, and Trichoptera (EPT) families were enumerated in 100 organism counts. A modified version of Petersen's (1992) Riparian, Channel, and Environmental (RCE) Inventory was used to assess habitat quality; benthic macroinvertebrate and fish observations were left out of this protocol, as the former were redundant with the RBP II survey and the latter could not be rapidly assessed. Single sediment samples were also collected from each stream by sieving surface sediments (top 10 cm) through a No. 10 U.S. Standard Sieve (2.00 mm mesh size). The sediment samples were then dried for 48 hours at 70 °C and disaggregated using a mortar and pestle. Between samples the mortar and pestle were cleaned with 10% v/v nitric acid and abrasion, rinsed, and dried in a drying oven. A 50 g portion of each sample was then digested in

200 ml of 10% v/v trace metals grade hydrochloric acid (Fisher Scientific) in acid washed polyethylene bottles for 96 hours at 70 °C (Bopp & Biggs, 1981); the portion of sediment metals solubilized by this treatment has been reported to be an environmentally active fraction. The samples were then filtered through 0.45 μm membrane filters, diluted appropriately in metal free 10% HCl, and analyzed using ICP by the Soil Testing and Plant Analysis Laboratory at Virginia Polytechnic Institute & State University. Metals not analyzed by ICP were analyzed by AAS using a Perkin Elmer 1100 Atomic Absorption Spectrophotometer, employing manufacturer recommended conditions. A total of seventeen elements (Ag, Al, As, Ca, Cd, Cr, Cu, Fe, Hg, Mg, Mn, Ni, Pb, S, Se, Sn, and Zn) were analyzed in the sediment digests. Contaminant metals (excluding Ca, Fe, Mg, Mn, and S) in each of the ten streams were defined as those which exceeded the mean concentration of a given metal in the five reference sites plus two standard deviations of the mean (Chester et al., 1985). The results of the three reconnaissance parameters (habitat quality, number of EPT families, and number of metal contaminants) were graphed in three dimensions and interpreted visually (Fig. 9).

Experimental Design and Data Collection

Chemical-releasing substrates were constructed by sealing unglazed, terra-cotta clay tiles over 10 cm x 10 cm square holes cut in 750 ml Falcon, polystyrene tissue culture flasks using silicone sealant (Dow Corning). Velcro was attached to the back of

the substrates using a waterproof epoxy resin. The completed substrates had an exposed tile area of 118 cm^2 and a total internal volume of 790 ml. Wooden pallets were secured to the bottom of the ten stream sites by packing stream cobbles into them. Pallets anchored in this manner remained in place for the duration of the experiment. On September 11, 1993, 25 labelled (i.e., pre-allocated to different treatment levels) chemical-releasing substrates per site were filled with distilled, deionized water (ddi H_2O) and randomly secured to velcro strips that had been tacked to the pallets prior to their placement in the streams. Substrate colonization was allowed to proceed in the absence of added copper stress for 21 days.

Samples were taken weekly in random order for the measurement of physicochemical parameters. Stream velocity was measured using a Flo-Mate model 2000 portable flowmeter (Marsh-McBirney, Inc.), and pH and temperature were measured using an Acumet 1003 portable pH meter and a KCl/AgCl probe with automatic temperature compensation (Fisher Scientific). Conductivity was measured with a YSI Model 33 S-C-T conductivity meter. Hardness and alkalinity were determined in unfiltered samples using EDTA and sulfuric acid titrations (endpoint pH=4.50), respectively (APHA et al., 1989). Concentrations of dissolved reactive orthophosphate, total phosphorus (digested with sulfuric acid and persulfate), ammonium, nitrite, and silica were analyzed colorimetrically using standard protocols (APHA et al, 1989); samples taken for total phosphorus were unfiltered, while the other analyses were performed on samples

filtered through 0.45 μm membrane filters. Chloride, nitrate, and sulfate concentrations were determined simultaneously in filtered (0.45 μm) water samples with a Dionex Series 2000i/SP ion chromatograph. Dissolved organic carbon (DOC) in water samples filtered through pre-ashed, GF/F glass fiber filters (0.7 μm; Fisher Scientific) was analyzed with a Dohrmann model DC-80 TOC Analyzer using UV/persulfate digestion.

After 21 days of colonization the periphyton communities growing on four baseline substrates (pre-labelled) were sampled from each stream with a stiff bristle toothbrush and a squirt bottle filled with filtered stream water. Removed periphyton slurries were brought up to a known volume in sample bottles, and a homogenized aliquot of each baseline sample was filtered onto a GF/F glass fiber filter and washed with 1% w/v disodium EDTA (pH=5.1) in the field to remove unbound copper from the community samples. The EDTA washed aliquots were transported to the laboratory on ice for analysis of baseline copper bioconcentration. The remaining periphyton homogenates were preserved in the field with M3 fixative lacking iodine (APHA et al., 1989). The biomass of dried bioconcentration subsamples (105 °C for 24 hours) was determined as loss of mass upon ashing at 500 °C for one hour (i.e., as ash-free dry mass, AFDM). Copper was extracted from the residual ash in 1 ml of concentrated, metals grade nitric acid (Fisher Scientific) for one week at room temperature. Acid washed glassware was used in all stages of this procedure. After acid digestion, the total volume of the extracts was brought up to 10 ml

with metal free water (5% final nitric acid concentration), the samples were centrifuged to remove particulate matter, and the copper in the supernatants was analyzed by AAS (graphite furnace atomization). Copper bioconcentration was reported as $mgCu \cdot gAFDM^{-1}$.

Immediately after taking baseline samples on day 21, the remaining substrates were removed from the pallets, emptied of the water they contained, filled with either ddi H_2O (i.e., controls) or varying concentrations of aqueous $CuCl_2 \cdot 2H_2O$, and replaced to their former positions on the substrates. Previous laboratory experiments (Arnegard, McCormick & Cairns, in press) demonstrated that copper flux across the chemical-releasing substrates and the exposure of periphyton to copper (measured as Cu bioconcentration) are both linear functions of the copper concentration in the modified flasks. This information allowed appropriate concentrations of $CuCl_2 \cdot 2H_2O$ to be chosen that would potentially generate toxic responses in the periphyton communities. Solutions having nominal copper concentrations of 0, 0.5, 1.0, 2.0, 4.0, and 6.0 $gCu^{2+} \cdot l^{-1}$ were each added to three chemical-releasing substrates per site. The third substrate receiving each copper solution, pre-labelled as such to avoid bias, served as a backup in the event of substrate loss. After filling all of the substrates at each site, samples were taken from the nominal copper solutions, treated with metal free nitric acid to yield a final acid concentration of 0.15% (APHA et al., 1989), diluted appropriately, and analyzed by AAS (flame atomization) to determine copper concentrations in the chemical-releasing substrates at the beginning of exposure phase.

In addition, triplicate, unfiltered water samples were taken 5 m upstream and 5 m downstream from the copper-releasing substrates at each site on day 4 of copper dosing to determine the potential impact of this method on the stream ecosystems. These samples were preserved with 0.15% metal free nitric acid, and total water column copper was determined by AAS (graphite furnace atomization).

After one week of receiving substrate-released copper under in situ conditions, two substrates per copper concentration (i.e, twelve substrates per site) were sampled in the same manner employed in baseline sampling. As before, copper exposure levels were measured as copper bioconcentration in 1% EDTA washed community aliquots, and the remaining periphyton slurries were fixed with modified M3. In addition, samples of the copper solutions remaining in the chemical-releasing substrates were removed, and metal free nitric acid was added to them (final concentration 15%). Copper concentrations in these samples were determined by AAS (flame atomization) to determine loss of copper during the week of exposure via diffusion through the terra-cotta tiles (i.e, copper flux across the chemical-releasing substrates). The concentrated copper solutions remaining in the flasks were then emptied into carboys and returned to the laboratory for processing as hazardous waste.

Community biomass was measured in aliquots of all baseline and copper exposed samples as ash-free dry mass (see above). In addition, permanent algal mounts were prepared for the quantification of community composition. Quantitatively diluted

subsamples for diatom enumeration were dehydrated by the vapor substitution method and mounted in HYRAX (Custom Research and Development, Auburn, CA) mounting medium (Stevenson & Stoermer, 1981). Duplicate subsamples were also mounted in Taft's Syrup Medium (Stevenson, 1984) to minimize distortion of soft algae and allow non-diatom algae to be identified and enumerated. The slide mounts were scanned at X1000 with a Nikon Microphot-FX microscope, using DIC microscopy when helpful for diatom identification. For both mount types, 500 specimens were identified (Pryfogle & Lowe, 1979) to the lowest possible taxon using standard taxonomic keys (Desikachary, 1959; Patrick & Reimer, 1966, 1975; Prescott, 1970, 1978; Germain, 1981; Dillard, 1989a, 1989b, 1990, 1991a, 1991b). Specimens that could not be identified to species were assigned numeric species labels on the basis of morphological differences. Reference drawings and photomicrographs were made to ensure consistent taxonomic classification. Diatoms displaying some remnant of protoplast in the HYRAX mounts and chlorophyll bearing diatoms in the TSM mounts were designated as being alive at the time of sample collection and were distinguished from dead (i.e., empty) frustules. Quantitative preparation of algal mounts allowed cell abundances to be measured per unit substrate area.

Data Analysis

Shannon-Weaver and Brillouin's diversity indices (Wilhm & Dorris, 1968) were calculated using per area species abundance matrices in the baseline and copper exposed samples. For the

exposure phase samples only, Euclidean distances and Pinkham-Pearson coefficients of similarity (Pinkham & Pearson, 1976) were calculated for each of two pairwise comparisons between each of twelve substrates and the two controls at each site. Only cells that were designated as alive were used in diversity and similarity calculations. These two parameters, along with per area biomass, diatom and total algal species richness (live cells in 500 cell counts), and the ratio of dead to living diatoms, comprised the structural response parameters. These responses were regressed against $\log_{10}$-transformed copper bioconcentration to determine the strength of the relationships between periphyton structure and copper exposure in each stream (Kleinbaum, Kupper & Muller, 1988; SAS Institute Inc., 1990). Copper bioconcentration was $\log_{10}$-transformed to linearize the response curves. Differences in structural responses to copper between communities in the four reference sites and communities in the four previously impacted sites were assessed using Analysis of Covariance (Kleinbaum, Kupper & Muller, 1988; SAS Institute Inc., 1990). This approach allowed testing of the null hypothesis: history of metal stress does not affect the current structural sensitivity of periphyton communities in southwestern Virginia streams to copper.

Results and Discussion

The data collected during this investigation is still being analyzed. The results that have already been obtained are discussed below.

Water and Habitat Quality of the Stream Ecosystems

Metal contamination, degraded stream habitat, and impaired biota were verified at the five streams chosen as impacted sites (Fig. 9). Upper layer sediments of all of the impacted streams contained one or more metal contaminants, as defined by Chester et al. (1985), relative to the sediments in the unimpacted streams, which contained no metal contaminants (Table 3). In general, concentrations of toxic metals in the surface layers of stream sediments are good indicators of periphyton exposure to these metals (Ramelow et al., 1992; Drndarski, Stojic & Markov, 1993). Relative to the reference sites, the modified Riparian, Channel, and Environmental (RCE) Inventory score (Petersen, 1992) was lower at all the impacted sites, reflecting more impaired stream habitats (Fig. 9). Generally, there were fewer Ephemeroptera, Plecoptera, and Trichoptera (EPT) families at the impacted sites than at the unimpacted sites (Fig. 9). The two exceptions to this were Kimberling Creek and Goose Creek. Information was subsequently obtained (see above) indicating that Kimberling Creek may have received metal inputs in the past. Macroinvertebrate sampling in Goose Creek yielded as few EPT families as the most taxa rich

impacted site, the North Fork Holston River. However, only a single sample was taken for this determination, so no conclusions on the robustness of the Goose Creek insect fauna can be made.

In many regards, the set of reference sites was chemically similar to the set of impacted sites (Table 4). The means of most of the measured physicochemical parameters were not significantly different between the five reference and the five impacted streams during the colonization phase of this investigation. Mean soluble silica was significantly lower in the impacted streams than in the reference streams, and mean water hardness was significantly higher in the impacted sites, due to the influence of two extremely hard water impacted streams, the Guest River and the North Fork Holston River. In addition, mean conductivity and mean concentrations of soluble nitrite, sulfate, and chloride were significantly higher in the group of impacted streams due to industrial inputs in some of these streams (Table 4).

Copper Flux and Periphyton Bioconcentration of Copper

Copper flux, calculated as the loss of copper from the chemical-releasing substrates during the seven day exposure phase, was a linear function of the internal copper concentration in all eight streams investigated (Fig. 10). Analysis of covariance revealed that the slopes of the linear regressions of copper flux against internal substrate copper concentrations were significantly different among the eight different streams ($p=0.0007$). The streams with the slopes of greatest magnitude tended to have the

hardest water and the swiftest velocities over the experimental pallets (Table 4).

The accumulation of copper by the periphyton communities was a significant linear function of copper flux at all sites except the Little River (Fig. 11). This indicates that a range of copper exposure levels can be established by the experimental substrates under a variety of field conditions. Communities in the Little River did show a structural response to copper exposure (see below), perhaps indicating experimental error in the determination of copper flux for this site. However, analysis of covariance revealed that both the intercepts ($p<0.0001$) and slopes ($p<0.0001$) of these linear relationships between copper bioconcentration and copper flux were significantly different among the eight streams studied. Long, visible algal filaments were noted at the two sites with the smallest slopes just before dosing and at the time of sample collection, whereas the periphyton communities in the other streams were more adnate in appearance. Thus, the physical structure of the periphyton communities may be an important factor affecting their exposure to substrate released copper. A gradient of copper concentrations is most likely established above the copper-releasing substrates, with the adnate forms being exposed to the highest concentration of copper and filamentous forms being exposed to relatively more dilute copper.

Periphyton Community Response to Copper

Although periphyton communities in seven of eight streams

bioconcentrated significant amounts of copper, community biomass in only three streams (the Clinch River, the Guest River, and the Little River) was a significant, negative linear function of $\log_{10}$-transformed copper bioconcentration (Fig. 12), including one stream in which bioconcentration itself was insignificant (the Little River). The regression of periphyton community biomass in Peak Creek against $\log_{10}$-transformed copper bioconcentration was marginally significant ($p=0.060$; Fig. 12).

Several possible explanations exist for the lack of significant responses of community biomass to copper bioconcentration in the four remaining streams. First, copper exposure may have been insufficient to elicit responses in these streams. Second, community adaptation or acclimation to metals, especially in the impacted streams, may have conferred copper resistance to periphyton communities in these streams. Third, after 21 days of colonization and community development, the periphyton communities may have reached a state of development in which natural sloughing begins to occur, irrespective of exposure to copper. Finally, significant loss of biomass may have occurred when the substrates were manipulated at the beginning of the seven day copper exposure phase in streams with loosely attached periphyton communities.

Some of the stream communities that failed to respond with decreased biomass also bioconcentrated the greatest amount of copper (e.g., Bottom Creek and Goose Creek communities). Inadequate copper exposure does not explain the lack of a

significant change in community biomass in these streams. However, insufficient copper exposure may explain the lack of response by communities in the North Fork Holston River, which bioconcentrated relatively little copper (Fig. 11). As discussed, communities in the North Fork Holston River were dominated by filamentous algae that may have been exposed to less copper due to their distance from the surface of the diffusing substrates.

The communities in Bottom Creek and Goose Creek were loosely attached to the substrates, and visible loss occurred during the manipulation required for filling the chemical-releasing substrates with copper solutions. Although all substrates, including the controls, were handled in the same way, the loss of community biomass during the experimental manipulation and resulting increase in variability in community biomass may have, in part, caused the lack of significant responses. Based on the sediment metals analysis, Peters Creek has been historically exposed to copper (Table 3). The lack of response in periphyton biomass to copper delivered to communities in Peters Creek may reflect the tolerance of periphyton in this stream to copper, although further investigation is needed to test this hypothesis.

Analysis of covariance of the data from only the sites yielding significant or marginally significant biomass responses, indicated that the y-intercepts of the response curves for the impacted sites (the Guest River and Peak Creek) are significantly greater ($p<0.0001$) than the y-intercepts for the reference sites (the Clinch River and the Little River). The slopes of the

responses in the two impacted sites are not distinguishable from the slopes in the reference sites ($p=0.213$). This reflects a greater periphyton biomass over all copper bioconcentrations in the two, more nutrient enriched impacted sites compared to the two, relatively oligotrophic reference sites. The hypothesis that the impacted periphyton communities are more or less resistant to further inputs of copper than the reference communities cannot be rejected, however, on the basis of indistinguishable biomass response slopes.

Of all the taxonomic structural measurements (Figs. 13 and 14), only diatom diversity in the North Fork Holston River communities was significantly depressed by exposure to copper. Periphyton biomass in this river was not significantly affected by copper (Fig. 12), perhaps due to the large contribution of one or more non-diatom algae to community biomass at this site. Investigation of this explanation awaits further analysis of the taxonomic data.

That periphyton community biomass was affected by copper in several streams in which the taxonomic structure was not significantly affected may have been a result of the nature of copper exposure. Because copper was delivered to the communities in a bottom-up fashion, the basal cells in the community were probably exposed to greater concentrations of copper. This may have caused entire patches of the community to slough off, reducing community biomass but leaving the remaining patches with overlying layers relatively intact taxonomically.

If further research supports this conclusion, a different experimental design with the chemical-releasing substrates is warranted. Instead of dosing colonized substrates with toxicant, in order to study its effects on developed periphyton communities, a better approach may be to initially place substrates that are already filled with toxicant solutions into streams, in order to investigate its effects on the community colonization and development processes. This would avoid the methodological problems (e.g., periphyton sloughing) associated with manipulating the substrates after community colonization has occurred. The high concentrations of copper left in the substrates after a week of diffusion indicate that toxicant diffusion can be sustained for a period of time long enough to allow for these types of studies to be conducted.

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TABLE 3. Results of the elemental analysis of stream sediments. Values are element concentrations in $mg \cdot kg^{-1}$ unless otherwise indicated. Artificial background sediment [ABS]** concentrations are means plus two standard deviations of the reference site data (including Kimberling Creek). Concentrations > [ABS], indicated by *, are considered contaminants. Detection limits were used in the calculation of [ABS] for elements below detection.

	SEDIMENT ELEMENT																
	Ag[c]	Al[b]	As[c]	Ca[d]	Cd[b]	Cr[b]	Cu[b]	Fe[b]	Hg[b]	Mg[d]	Mn[b]	Ni[b]	Pb[c]	S[b]	Se[b]	Sn[c]	Zn[b]
	$\mu g \cdot kg^{-1}$	$g \cdot kg^{-1}$		$g \cdot kg^{-1}$				$g \cdot kg^{-1}$		$g \cdot kg^{-1}$							
Reference Streams																	
B	3.4	4.3	3.8	0.2	0.8	7.6	4.2	9.1	0.9	0.9	242	5.8	11	26	b.d.	0.32	26
C	12	2.1	2.0	60	1.0	12	4.2	7.4	0.9	3.1	760	7.0	20	65	b.d.	b.d.	28
Go	13	5.2	4.1	1.4	0.8	14	50	10	2.4	2.5	235	8.1	14	28	b.d.	b.d.	48
K	2.8	8.2	7.1	0.2	2.2	24	36	30	3.4	1.9	667	41	31	59	b.d.	b.d.	103
L	b.d.	2.6	3.7	30	1.2	20	4.9	13	0.8	1.8	620	7.7	14	50	b.d.	1.6	28
[ABS]**	17	9.3	7.8	n.c.	2.3	28	63	n.c.	4.0	n.c.	n.c.	45	34	n.c.	n.c.	1.8	112
Impacted Streams																	
Gr	b.d.	4.6	11*	0.1	1.4	12	38	16	1.7	1.0	182	12	19	56	b.d.	0.60	38
Gu	28*	2.4	2.8	0.5	0.8	5.9	54	8.8	-	0.7	1088	17	20	77	b.d.	9.6*	54
H(1)[a]	1.5	0.1	b.d.	87	0.5	2.1	26	0.5	0.2[e]	1.2	224	2.1	3.6	72	b.d.	b.d.	26
H(2)[a]	1.7	0.05	b.d.	92	0.5	2.1	6.4	0.2	0.3[e]	1.2	242	2.0	2.3	99	b.d.	b.d.	6.4
Pk	81*	8.9	5.6	5.4	2.0	16	2183*	24	3.4	5.2	641	28	228*	337	b.d.	5.6*	2183*
Pt	78*	3.3	2.9	34	2.0	88*	146*	10	1.1	15	2930	17	64*	77	b.d.	b.d.	146*

**Defined by Chester et al. (1985) and explained in the text.
[a]A second N. Fk. Holston sample was taken to corroborate the results of the first sample.
[b]Inductively Coupled Plasma (ICP) Emission Spectroscopy.
[c]Atomic Absorption Spectrometry (furnace atomization).
[d]Atomic Absorption Spectrometry (flame atomization).
[e]Other sources (see text) indicate that Hg is currently a contaminant at the Holston site.
b.d. = below the analytical detection limit.
n.c. = [ABS] not calculated; element not particularly toxic or data not adequate.
- = missing datum.

TABLE 4. Physical and chemical characterization of the streams. Table entries are means, standard errors, and numbers of observations. Overall means of reference and impacted sites marked with ** are significantly different from one another ($p<0.05$).

	Reference Streams				Overall	
Parameter	B	C	Go	L	Mean	K(excluded)
Temperature	15.5	17.1	16.6	16.0	16.3	14.1
(°C)	2.0	2.0	2.8	1.6	0.9	1.3
	4	4	3	4	15	4
Velocity	0.06	0.27	0.11	0.17	0.15	0.01
($m \cdot s^{-1}$)	-	-	-	-	0.04	-
	1	1	1	1	4	1
pH	7.51	8.35	7.95	8.39	8.05	6.99
(units)	0.31	0.26	0.17	0.12	0.14	0.03
	3	3	3	3	12	3
Conductivity	75.7	327	75.7	229	177**	368
($\mu S \cdot cm^{-2}$)	6.0	31	4.3	8	33	56
	3	3	3	3	12	3
Hardness	37.0	167	38.2	143	96.3**	104
($mg \cdot l^{-1}$	3.0	1	2.9	2	17.9	29
as $CaCO_3$)	3	3	3	3	12	3
Alkalinity	24.6	125	28.6	108	71.4	16.3
($mg \cdot l^{-1}$	0.9	2	0.8	2	13.6	0.3
as $CaCO_3$)	3	3	3	3	12	3
DOC	1.87	2.20	1.67	1.62	1.84	0.92
($mg \cdot l^{-1}$)	0.41	0.10	0.22	0.05	0.12	0.04
	2	2	2	2	8	2
Total-P	28.7	60.1	28.2	39.1	39.0	9.6
($\mu g \cdot l^{-1}$)	9.5	15.2	4.7	11.4	6.0	4.8
	3	3	3	3	12	3
o-phosphate-P	11.3	54.0	10.2	12.7	22.1	1.8
($\mu g \cdot l^{-1}$)	3.7	10.6	3.2	3.1	6.1	1.8
	3	3	3	3	12	3
NH_4^+	4.5	10.7	6.2	17.3	9.7	5.9
($\mu g \cdot l^{-1}$)	4.0	6.5	3.2	8.7	3.0	4.1
	3	3	3	3	12	3
NO_2^-	2.3	8.8	2.1	9.5	5.7**	0.2
($\mu g \cdot l^{-1}$)	0.5	1.8	0.2	1.1	1.1	0.2
	3	3	3	3	12	3
NO_3^-	1.34	4.22	0.45	3.74	2.44	0.49
($mg \cdot l^{-1}$)	0.05	0.27	0.22	0.02	0.48	0.07
	3	3	3	3	12	3
SO_4^{2-}	3.39	10.3	2.65	4.04	5.10**	68.5
($mg \cdot l^{-1}$)	0.74	1.0	0.38	0.18	0.96	6.4
	3	3	3	3	12	3
Cl^-	4.34	4.09	2.03	1.24	2.93**	22.3
($mg \cdot l^{-1}$)	1.12	0.17	0.15	0.33	0.47	4.9
	3	3	3	3	12	3
SiO_2	8.5	5.0	9.4	4.3	6.79**	6.1
($mg \cdot l^{-1}$)	0.6	0.6	1.9	0.3	0.80	0.9
	3	3	3	3	12	3

TABLE 4, continued

Parameter	Impacted Streams Gu	H	Pk	Pt	Overall Mean	Gr(flooded)
Temperature	17.1	18.8	18.6	18.5	18.3	18.8
(°C)	1.6	1.6	1.6	1.6	0.7	0.4
	4	4	4	4	16	2
Velocity	0.13	0.22	0.28	0.04	0.17	-
($m \cdot s^{-1}$)	-	-	-	-	0.05	-
	1	1	1	1	4	-
pH	8.22	8.31	7.76	7.79	8.02	7.29
(units)	0.30	0.19	0.06	0.44	0.14	0.05
	3	3	3	3	12	2
Conductivity	767	957	1399	678	950**	232
($\mu S \cdot cm^{-2}$)	82	30	713	274	184	20
	3	3	3	3	12	2
Hardness	329	267	81.1	181	215**	44.0
($mg \cdot l^{-1}$	17	9	9.4	8	28	0
as $CaCO_3$)	3	3	3	3	12	2
Alkalinity	110	98.2	51.9	130	97.6	12.6
($mg \cdot l^{-1}$	4	3.6	4.6	3	8.8	0
as $CaCO_3$)	3	3	3	3	12	2
DOC	3.98	2.11	2.70	9.97	4.69	-
($mg \cdot l^{-1}$)	0.70	0.13	0.04	3.55	1.36	-
	2	2	2	2	8	-
Total-P	41.0	17.0	125	24.6	51.9	35.1
($\mu g \cdot l^{-1}$)	6.0	2.4	36	1.5	15.2	8.6
	3	3	3	3	12	2
o-phosphate-P	16.1	5.1	84.9	4.8	27.7	10.0
($\mu g \cdot l^{-1}$)	7.2	2.6	30.5	2.5	12.1	1.0
	3	3	3	3	12	2
NH_4^+	28.2	17.2	56.4	33.9	33.9	1888
($\mu g \cdot l^{-1}$)	21.1	8.1	44.2	12.6	11.7	688
	3	3	3	3	12	2
NO_2^-	14.2	9.2	10.5	38.7	18.2**	96.3
($\mu g \cdot l^{-1}$)	2.9	2.6	0.9	13.9	4.8	90.7
	3	3	3	3	12	2
NO_3^-	2.19	1.22	0.30	2.53	1.56	1.60
($mg \cdot l^{-1}$)	0.59	0.50	0.03	0.29	0.32	0
	3	3	3	3	12	2
SO_4^{2-}	284	48.1	1186	24.6	386**	34.7
($mg \cdot l^{-1}$)	23	6.8	415	10.8	168	14.7
	3	3	3	3	12	2
Cl^-	25.9	102	28.2	24.6	45.3**	13.8
($mg \cdot l^{-1}$)	12.1	13	17.0	13.8	11.6	12.6
	3	3	3	3	12	2
SiO_2	4.0	2.0	5.3	7.2	4.62**	3.4
($mg \cdot l^{-1}$)	0.4	0.8	0.8	0.8	0.65	-
	3	3	3	3	12	1

FIGURE 8

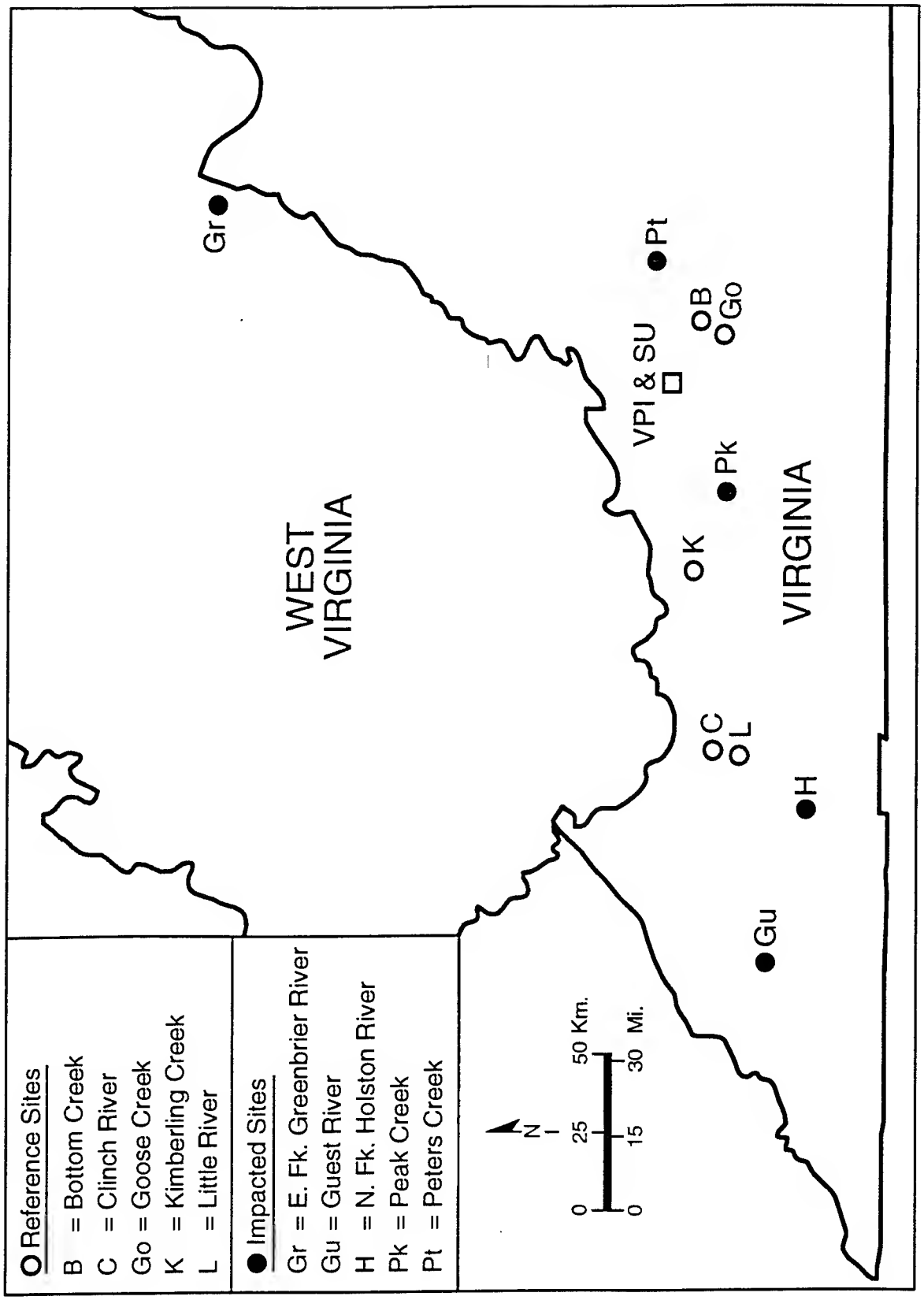
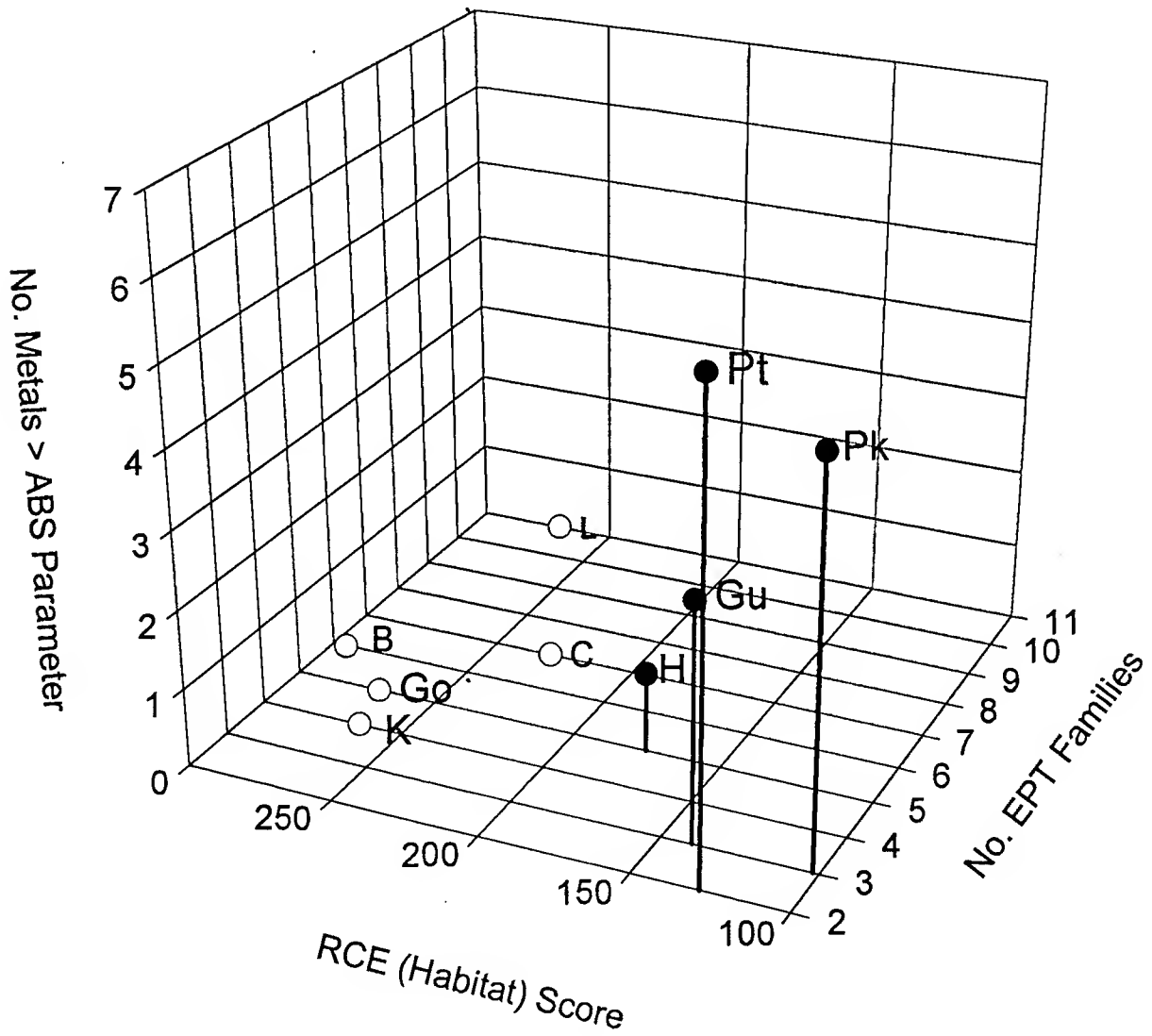

Reference Sites
B = Bottom Creek
C = Clinch River
Go = Goose Creek
K = Kimberling Creek
L = Little River
Impacted Sites
Gr = E. Fk. Greenbrier River
Gu = Guest River
H = N. Fk. Holston River
Pk = Peak Creek
Pt = Peters Creek
WEST VIRGINIA
VIRGINIA
Gr
Pt
B
Go
VPI & SU
K
Pk
C
L
H
Gu
N
0
25
50 Km.
0
15
30 Mi.

FIGURE 9

Habitat Quality, No. EPT Families, No. Sediment Metal Contaminants



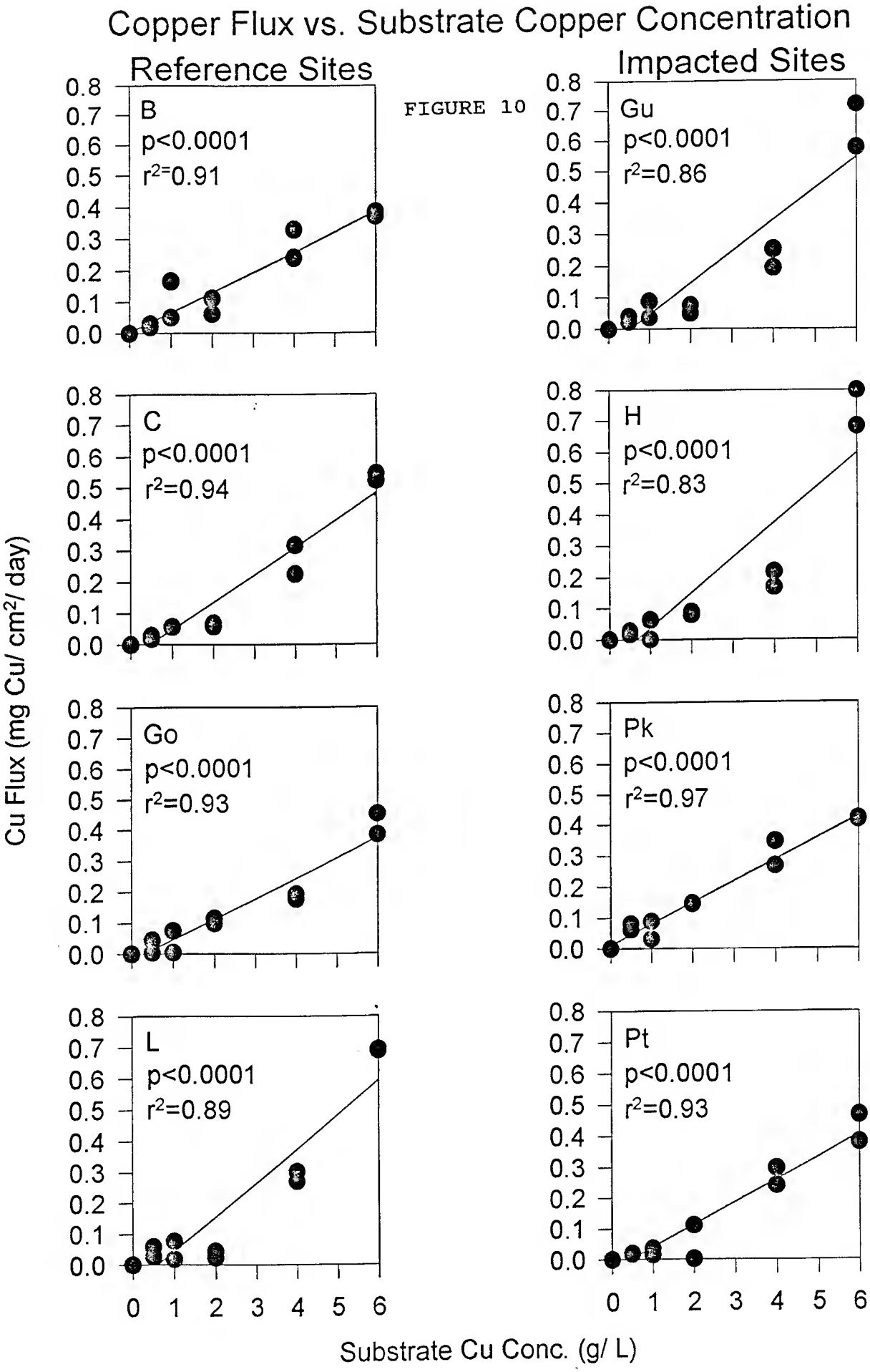
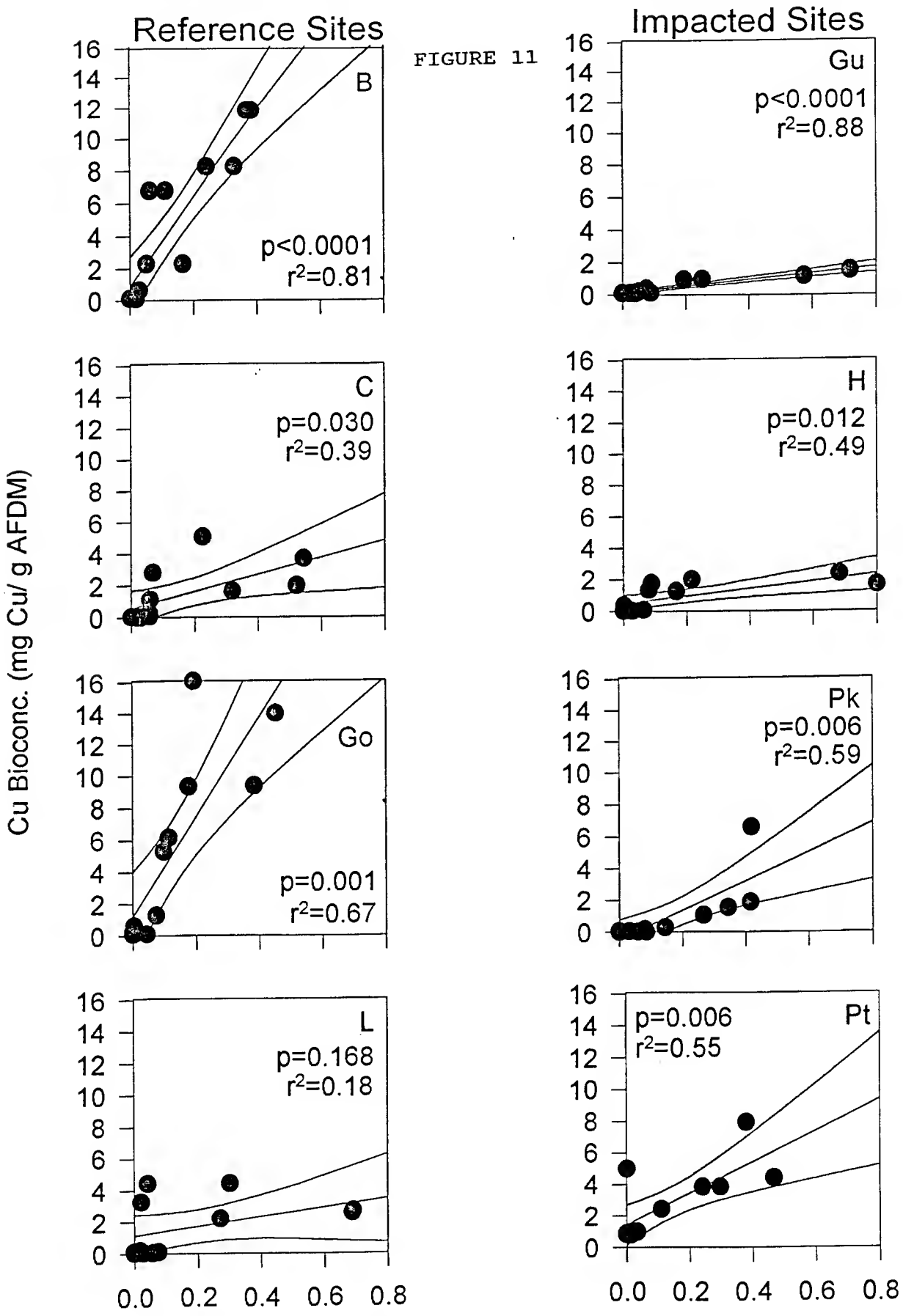

Copper Flux vs. Substrate Copper Concentration
Reference Sites
Impacted Sites
B
p<0.0001
r²=0.91
Gu
p<0.0001
r²=0.86
C
p<0.0001
r²=0.94
H
p<0.0001
r²=0.83
Go
p<0.0001
r²=0.93
Pk
p<0.0001
r²=0.97
L
p<0.0001
r²=0.89
Pt
p<0.0001
r²=0.93
Cu Flux (mg Cu/ cm²/ day)
Substrate Cu Conc. (g/ L)

FIGURE 10

Copper Bioconconcentration vs. Copper Flux

FIGURE 11



AFDM vs. Copper Bioconcentration

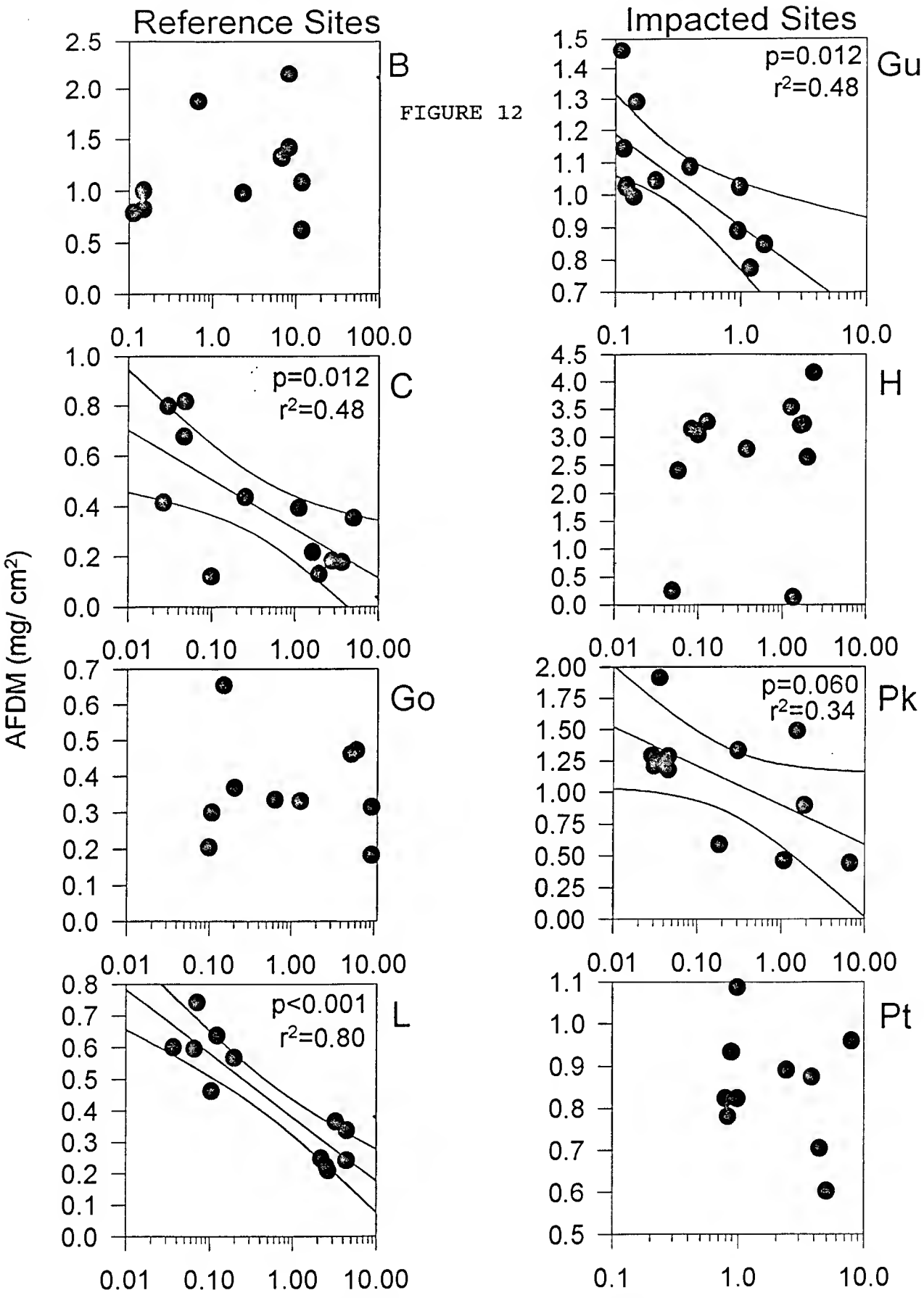


FIGURE 12

FIGURE 13

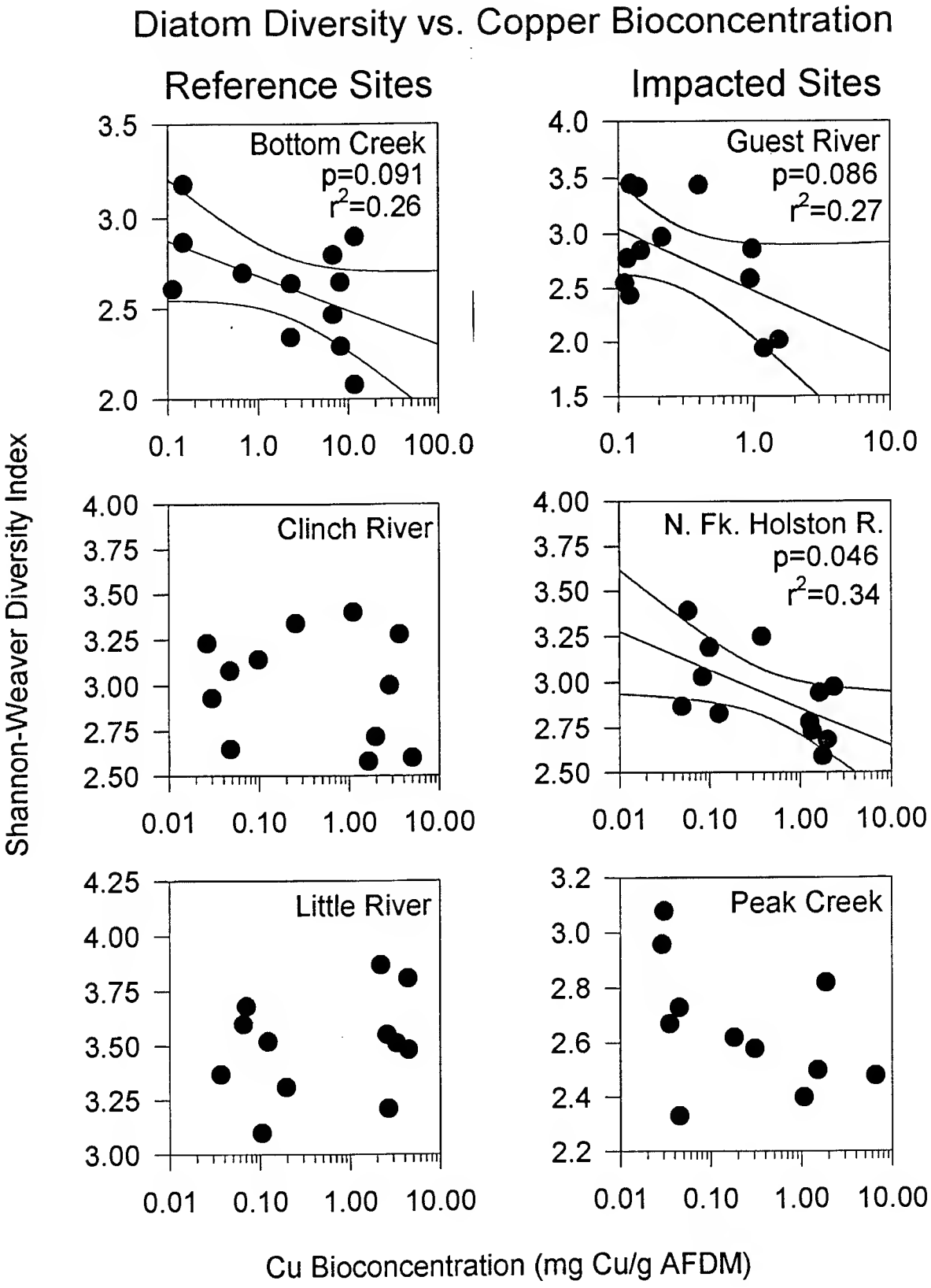
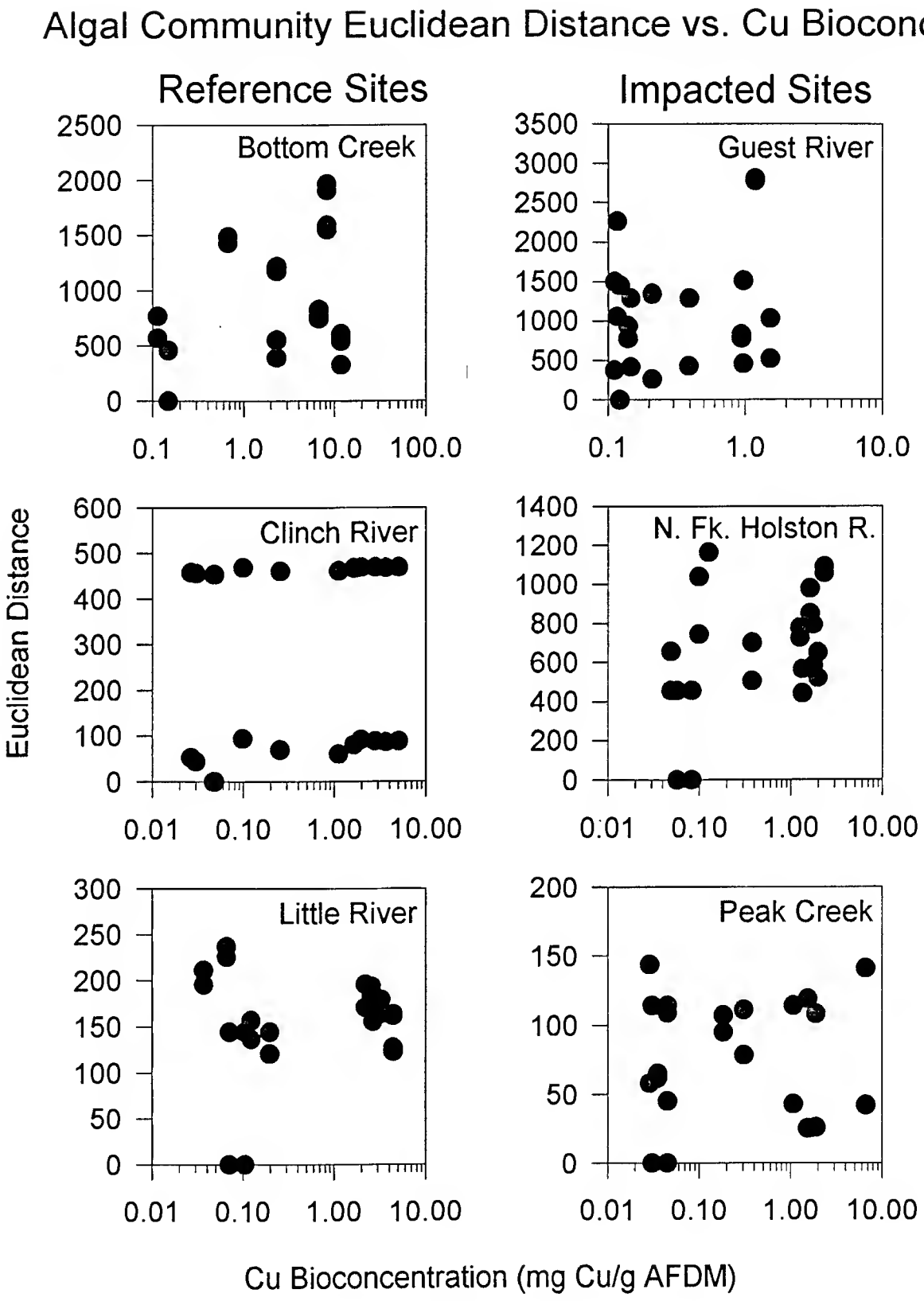

Diatom Diversity vs. Copper Bioconcentration
Reference Sites
Impacted Sites
Bottom Creek
p=0.091
r2=0.26
Guest River
p=0.086
r2=0.27
Clinch River
N. Fk. Holston R.
p=0.046
r2=0.34
Little River
Peak Creek
Shannon-Weaver Diversity Index
Cu Bioconcentration (mg Cu/g AFDM)

FIGURE 14


Algal Community Euclidean Distance vs. Cu Bioconc.
Reference Sites
Impacted Sites
Bottom Creek
Guest River
Clinch River
N. Fk. Holston R.
Little River
Peak Creek
Euclidean Distance
Cu Bioconcentration (mg Cu/g AFDM)

CONCLUSIONS

This research has resulted in the development of chemical-releasing substrates that allow periphyton community toxicity tests to be conducted *in situ* in lotic ecosystems without adversely affecting the stream ecosystems under investigation. The porous terra-cotta colonization surface of these substrates is permeable to a variety of inorganic toxicants that can potentially be used in experiments with toxicant-releasing substrates. One significant advantages of this approach is that toxicants can be delivered to periphyton communities under natural environmental conditions, while undetectable amounts of toxicant are released to the stream ecosystems themselves.

Laboratory studies revealed that the non-taxonomic structural responses of periphyton communities exposed to substrate-released copper closely correspond to those obtained via the standard toxicant delivery method of adding copper to the water column of the test systems (i.e., artificial streams). The responses of the periphyton communities to copper stress delivered in both of the manners used in these studies were similar to those reported in the literature. However, these studies revealed a methodological artifact that could potentially affect the outcome of future investigations. Protocols used to sample periphyton from the toxicant-releasing substrates by abrasion may remove small amounts of clay from the tiles employed in the experimental substrates, and

this material may have strongly bound the toxicant being diffused. Subsequent periphyton digestion methods used to estimate toxicant bioconcentration inevitably incorporate the removed clay, thus, overestimating toxicant exposure. This effect was found to be very minor in the laboratory validation studies conducted with copper, but steps should be taken to minimize it in the future. For example, periphyton should be sampled from these substrates using less abrasive techniques, and/or the sampled periphyton should be decanting away from the heavier clay particles.

Toxicant-releasing substrates have many potential applications. Because they are used in situ, toxicant-releasing substrates may be used to validate laboratory toxicological studies under natural field conditions. The results of upstream-downstream investigations compare polluted regions of a stream to upstream reference sites. In addition to differences in toxicant exposures, differences in ecological characteristics (e.g., water velocity, substrate type, and incident solar radiation) weaken the conclusions drawn from such studies. Investigations utilizing toxicant-releasing substrates may be conducted at a single site within a stream, avoiding such problems. In addition, periphyton sensitivity to toxicants may be investigated using toxicant-releasing substrates before the occurrence of industrial inputs at a given site, yielding information of value to regional industrial development planners. Finally, toxicant-releasing substrates provide one of the only means of assessing the variability of natural periphyton community sensitivities to toxicants and the

role that history of stress plays in modifying periphyton sensitivity to stress.

A preliminary field investigation was conducted with the toxicant-releasing substrates. This study was aimed at investigating the differences in copper sensitivities of periphyton communities in relatively pristine and metal impacted streams. The hypothesis that periphyton communities in impacted streams respond to copper additions differently than communities in unimpacted streams could not be rejected, due largely to additional methodological problems associated with toxicant-releasing substrates. Communities that are loosely attached to the substrates may loose significant biomass during the manipulations required to fill these substrates with toxicant at the beginning of the exposure period. In addition, these substrates deliver toxicant in a bottom-up fashion. Entire patches of the periphyton community, including less impacted overlying algae that are exposed to lower concentrations of toxicant, may be lost from the substrates as the basal cells die and slough.

Toxicant-releasing substrates that are filled with the same concentrations of copper chloride diffuse copper at different rates, and periphyton communities exposed to the same rates of copper diffusion bioconcentrate different amounts of copper. A gradient of copper concentrations is probably established over the toxicant-releasing substrates, with the highest concentrations experienced by adnate algae and the lowest concentrations experienced by long filaments. Thus, the physical structure of

periphyton communities may be an important factor in the differential bioconcentration of copper released from the experimental substrates.

To overcome these problems in the future, toxicant solutions should be added to the substrates before they are initially placed in the streams rather than after a period of colonization under no stress. The focus of such studies would be on the effects of toxicants on the colonization and development of periphyton communities, rather than on the effects of toxicants on mature communities. Younger communities tend to be composed of relatively more adnate algal forms, as filaments require longer to grow. In addition, the recommended approach would not require the adverse manipulations needed to fill the substrates with toxicant after algal colonization and before the dosing period. Finally, this method may not be applicable in streams in which significant amounts of fine particular organic matter settle out on the substrates, preventing the formation of firmly adherent communities.

LIST OF MANUSCRIPTS, PRESENTATIONS AND SUPPORTED DEGREES

Manuscripts

Arnegard, M. E., P. V. McCormick, and J. Cairns, Jr. In prep. Resistance and resilience of stream periphyton assemblages to pH stress. To be submitted to Aquatic Toxicology.

McCormick, P. V., M. E. Arnegard, and J. Cairns, Jr. Submitted. A field-based method for quantifying the response of benthic stream communities to chemical stressors. Journal of Aquatic Ecosystem Health.

McCormick, P. V., M. E. Arnegard, and J. Cairns, Jr. In prep. Assessing regional variability in ecosystem sensitivity to anthropogenic stress: Stream periphyton responses to chlorine exposure. To be submitted to Ecological Applications.

McCormick, P. V., and J. Cairns, Jr. Submitted. An evaluation of the use of algal indicators for water quality assessments. Journal of Applied Phycology.

Presentations

McCormick, P. V., M. E. Arnegard, and J. Cairns, Jr. 1993. An in situ experimental method for assessing aquatic ecosystem responses to chemical stressors. Abstracts of the Third International Conference of Aquatic Ecosystem Health & the Ecological Significance of Bioassay Techniques, VPI&SU, Blacksburg, Virginia.

Arnegard, M. E., J. Cairns, Jr., E. E. Schiedt, and J. M. McMunigal. 1993. Comparison of the responses of periphyton communities to copper delivered either through the water column or via chemical-releasing substrates. Virginia Journal of Science. Presented at the 71st Annual Meeting of the Virginia Academy of Science, Old Dominion University, Norfolk, Virginia.

Arnegard, M. E., P. V. McCormick, and J. Cairns, Jr. 1992. A new method for assessing the effects of toxicants on Aufwuchs communities in situ using chemical-diffusing substrates. NABS Bulletin, Vol. 9, No. 1. Presented at the 40th Annual North American Benthological Society Meeting, University of Louisville, Louisville, Kentucky.

Arnegard, M. E. and J. Cairns, Jr. 1992. A new method for assessing the effects of inorganic pollutants on algal communities in streams. Proceedings of the Third Annual Environment Virginia Symposium. pp. 42-46. Presented at the Third Annual Environment Virginia Symposium, VMI, Lexington, Virginia.

Arnegard, M. E., P. V. McCormick, and J. Cairns, Jr. 1991. The development of chemical-diffusing substrates for in situ periphyton community surveys. Virginia Journal of Science, Vol. 42, No. 2. Presented at the 69th Annual Meeting of the Virginia Academy of Science, VPI&SU, Blacksburg, Virginia.

Supported Degrees

Arnegard, M. E. 1993. Toxicant-Releasing Substrates: A New Method for Delivering Copper to Microbial Communities in situ. Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Master of Science in Biology. 131 pp.